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UNIVERSITY OF ALBERTA

Fungal Biotransformation of Xenobiotics.

By

Derrick G. Freitag ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

In

Pharmaceutical Sciences

(Drug Metabolism)

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton Alberta

Spring 1995

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Dedication

This work is dedicated to Glenda for her love, encouragement, and patience and to my parents the best teachers one could ask for.

Abstract

To ensure the safety and effectiveness of a new therapeutic agent, it must be thoroughly investigated before approval can be given for human use. One component of this research is to evaluate the manner in which a drug is metabolized and whether the metabolites have activity or toxicity. Traditionally, this information has been obtained from various animal models. However, with growing concern over animal testing, efforts are being made to find acceptable alternative or complementary techniques.

For preliminary drug metabolism investigations, a viable complement to animal studies is to utilize microorganisms which have demonstrated the ability to metabolize drugs in a fashion similar to humans. More specifically, fungi have proven to be a good model for oxidative biotransformations because they possess a cytochrome P_{450} enzyme system analogous to that in humans. Fungi can be used to generate a metabolic profile of the possible human oxidative metabolites and their biosynthesis can be scaled up for further biological evaluation.

This fungal model was used to evaluate the metabolism of *rac*-mexiletine, *rac*-propranolol, and deuterated *rac*-propranolol *in vitro.* It was determined that the fungus *Cunninghamella echinulata* was capable of biosynthesizing several human metabolites from these drugs and their production was significantly affected by growth media composition and culture conditions. In addition, the metabolism of *rac*-mexiletine was found to be stereoselective. Finally, it was possible to biosynthesize deuterated metabolite(s) from isotopically labeled deuterated *rac*-propranolol.

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Even though this thesis will carry one author's name, the work contained within could not have been accomplished without the contribution of many different people. I would like to acknowledge the following individuals who helped make this work possible. To Franco Pasutto for giving me the opportunity to be a graduate student, when no one else would, and for showing me how to think like a scientist. To Ron Coutts whose excitement for research and teaching was contagious. To Bob Foster for teaching me the art of HPLC. To Len Wiebe for providing me with the opportunity to teach and play with radio labels. To Michael Pickard for showing me how to work with temperamental fungi. To Don Whyte for being able to fix and design anything despite having more work than time. To the Faculty of Pharmacy for providing me with funding and giving me the opportunity to learn and teach. To Brian Parasiuk for giving me my first teaching job. To Cathy Lemko whose common sense and encouragement helped me working through numerous analytical problems. To Gordon Haverland for all those wacky ideas. Finally, to Rudi Ladenius for being a good friend.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection.
AHMM	N-Acetyl-hydroxymethylmexiletine.
AMEX	N-Acetyl-mexiletine.
APHM	N-Acetyl-p-hydroxymexiletine.
ATOC	N-Acetyl-mexiletine.
Biohood	Biohazard containment hood.
CNS	Central nervous system.
Conc.	Concentration.
CV	Coefficient of variation.
DMAP	4-Dimethylaminopyridine.
DIP	N-Desisopropylpropranolol.
DNA	Deoxyribonucleic Acid.
cDNA	Complimentary Deoxyribonucleic Acid.
DTT	Dithiothreitol.
EDTA	Ethylenediamine tetraacetic acid.
EM	Extensive metabolizer.
g	Gram.
HEPES	N, 2-Hydroxyethylpiperazine N′-ethane sulfonic acid.
HPLC	High performance liquid chromatography.
HMM	Hydroxymethylmexiletine.
4HOP	4-Hydroxypropranolol.
4HOPG	4-Hydroxypropranolol glucuronide.
4HOPS	4-Hydroxypropranolol sulfate.
L	Liter.
mL	Milliliter.
mg	Milligram
MOPS	3-(N-morpholino) propanesulfonic acid.
NADPH	Nicotinamide adenine dinucleotide phosphate.

NEIC	S-(+)-1-(1-Naphthyl)ethyl isocyanate.
N-HMM	N-Hydroxy-hydroxymethylmexiletine.
NLA	Naphthoxylactic acid.
N-PHM	N-Hydroxy-p-hydroxymexiletine.
PMSF	Phenylmethylsulfonyl fluoride.
PM	Poor metabolizer.
PRL	Prarie Regional Laboratories.
Q. C.	Quality control.
R	Rectus.
rac	Racemic.
S	Sinister.
Stdev	Standard deviation.
Tris	Tris(hydroxymethyl)methylamine.
µg	Microgram.
µL	Microliter.
x g	Times gravity.

1.0 Introduction

Through diet and lifestyle, we interact with an environment that introduces us to hundreds of unique organic and inorganic compounds. Many of these, including food additives, pollutants, and drugs, have the potential to accumulate in various tissues if they succeed in entering the body. Here they can exert a beneficial effect or become a potential for harm. Mammals have responded to these challenges by adapting enzymes, normally associated with the metabolism of endogenous constituents, to process foreign chemicals (xenobiotics) and encourage their elimination. This "elimination" process was originally termed detoxication (Williams 1947) after it was assumed that a xenobiotic was transformed into a metabolite less toxic than the parent molecule. However, when *in vivo* activity was examined closely it was discovered that metabolism could generate compounds with elevated activity, or increased toxicity (Drayer 1982). This demonstrated a direct relationship between biotransformation and pharmacological activity underscoring that a xenobiotic cannot be considered a stable entity that enters the body and generates a specific response.

The number of unique chemical entities discovered or synthesized following the industrial revolution has steadily increased. Evidence of this growth can be viewed in the American Chemical Society's Chemical Abstract Registry which lists over four million distinct molecules. The possibility of exposure to one of these chemicals and its access to the bloodstream is likely. As a result, mechanisms must be in place to facilitate elimination. Synthesizing a unique enzyme as a direct response to each xenobiotic detected is one approach to defending the body against chemical challenges. The immune system neutralizes pathogenic organisms and foreign proteins in this manner. By utilizing nonspecific enzymes, our body has developed an alternative strategy to deal with xenobiotics. These enzymes are versatile enough to metabolize the majority of chemicals encountered. However, this creates a problem when attempting to predict activity and routes of elimination for new molecules placed in a biological system. Biotransformation can have a number of possible outcomes which must be determined experimentally. Therefore, when new molecules are designed for human consumption their effectiveness must be considered in conjunction with the activity of all metabolites before being considered safe for human use.

Traditionally, information regarding the manner in which drugs are metabolized has been obtained from various animal models. However, growing concern over animal testing has prompted the investigation of alternative techniques. One approach developed was to utilize microorganisms that demonstrated an ability to metabolize drugs in a fashion similar to humans (Smith and Rosazza 1975, Clark *et al.* 1991). Our objective was to continue this work by illustrating how microbial models could be utilized for preliminary drug metabolism studies. In general, their usefulness resides with their ability to generate a metabolic profile of the possible oxidative human metabolites. Furthermore, metabolite biosynthesis can be scaled up. In addition, by using isotopically labeled drug substrates (semi) preparative amounts of labeled metabolite may become readily available. Even though there are certain advantages to using microorganisms, they cannot replace traditional animal models for drug testing. Nonetheless, we hope to demonstrate that microbial models can provide useful information for preliminary drug investigations when compared to other *in vitro* techniques.

1.1 Mammalian Drug Metabolism

1.1.1 Introduction

After a drug is released into the body, it must maneuver through numerous obstacles before reaching its site of action. For example, if a drug is administered orally, it must pass through the acidic stomach where hydrolysis can occur. From here it will enter the gastrointestinal tract which contains various digestive enzymes and microorganisms. These can degrade a drug or alter its structure, preventing it from having a therapeutic benefit even before it reaches the blood stream (Kaminsky and Fasco 1992).

In general, if a drug can avoid these chemical-physical barriers, absorption will occur in the villi of the small intestine. The drug is then transported to the liver by the portal circulation where it may encounter hepatic enzymes (first pass metabolism). These enzymes are capable of metabolizing a foreign compound before it passes through to the rest of the body (Tam 1993). Even with these additional obstacles, many drugs will enter the general circulation without a significant loss in activity. Supplementary systems are also in place to help eliminate compounds. Drugs can bind to plasma proteins (Pacifici and Viani 1992), they can be stored in bone or fat tissue (Balant and Gex-Fabry 1990), and they can be eliminated by the kidneys and excreted into the urine (Weiner 1967). Therefore, a number of nonspecific as well as specific mechanisms are in place to protect the body from foreign chemicals.

1.1.2 Pathways of Drug Metabolism

Mechanisms that prevent a drug from reaching its site of action were briefly described in the previous section. They were discussed in order to emphasize that the body is not limited in the number of techniques it can use to prevent foreign compounds from accumulating. However, from this point forward only the enzymatic pathways utilized to process drugs and xenobiotics will be discussed. There has been considerable interest in this topic because the outcome of drug metabolism can be difficult to predict. A drug can be converted into a metabolite with less pharmacological activity than the parent compound. A drug can also be transformed into an active metabolite whose activity is greater than, or different from, the parent drug. Therefore, a tremendous amount of research has focused on the area of drug metabolism and the underlying mechanisms responsible for this uncertainty.

The pathways of drug biotransformation can be divided into two major categories (Williams 1967). The first is called phase I metabolism; its purpose is to introduce or expose a polar functional group on a drug by oxidative, reductive, or hydrolytic methods. Also known as functionalization, this process increases a drug's polarity and water solubility, thus enhancing excretion. Products from phase I will act as substrates for phase II metabolism, which is known as conjugation. Phase II reactions attach polar endogenous compounds to reactive functional groups. This is intended to increase the molecule's water solubility, prevent tubular resorption by the kidneys, and generate products with decreased activity or toxicity. A summary of phase I and II reactions are listed in table 1-1.

Table 1-1. Summary of the pathways involved in mammalian drug metabolism (Low and Castagnoli 1991).

Phase I Reactions (Functionalization)	**Phase II Reactions** (Conjugation)
Oxidations	Glucuronide formation
Hydroxylation - Aliphatic	Sulfate formation
- Aromatic	Acetylation
Dealkylation	Glycine conjugation
Deamination	Methylation
Reductions	Glutathione conjugation
Aldehyde reduction	
Ketone reduction	
Hydrolysis	
Deesterification	

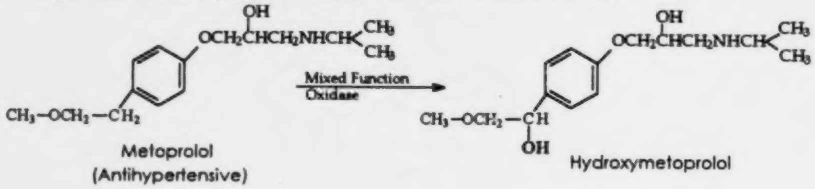
1.1.3 Oxidative Metabolism

Of the phase I reactions, oxidation is probably responsible for the largest quantity of metabolites formed during mammalian drug metabolism. Most oxidative metabolism takes place in the liver by a versatile group of enzymes referred to as mixed function oxidases or monooxygenases (Williams 1971).

They are nonspecific in nature which allows them to oxidize a variety of structurally diverse substrates (Table 1-2). They function by activating molecular oxygen and incorporating one atom into an organic substrate with the concomitant reduction of the other atom to water. Overall, two substrates are required as reductants of the two oxygen atoms in O_2. The principle substrate (drug) will accept one oxygen atom and a co-substrate will furnish the hydrogen

Table 1-2. Examples of oxidative reactions performed by the mixed function oxidase enzyme system.

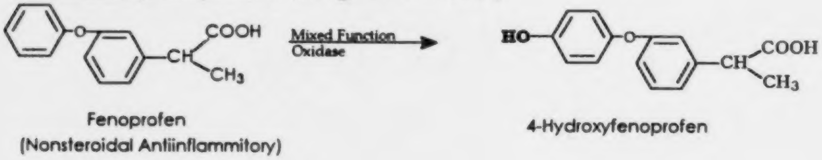
Aliphatic Hydroxylation (Regardh and Johnsson 1980)



Metoprolol (Antihypertensive)

Hydroxymetoprolol

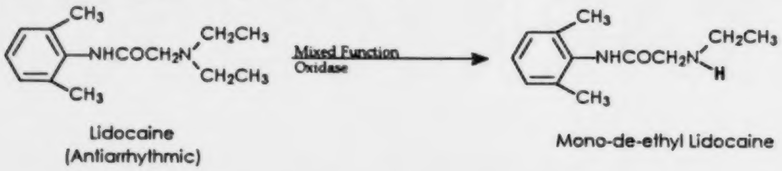
Aromatic Hydroxylation (Brogden *et al.* 1977)



Fenoprofen (Nonsteroidal Antiinflammitory)

4-Hydroxyfenoprofen

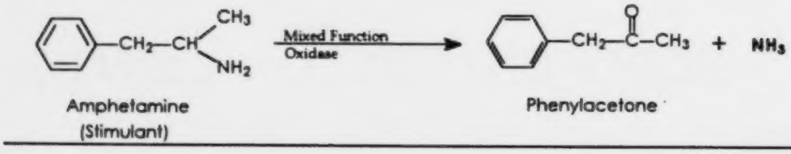
Dealkylation (Keenaghan *et al.* 1972)



Lidocaine (Antiarrhythmic)

Mono-de-ethyl Lidocaine

Deamination (Axelrod *et al.* 1955)



Amphetamine (Stimulant)

Phenylacetone

atom needed to reduce the other oxygen atom to water (White and Coon 1980). The stoichiometry that describes this oxidation can be written as:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

1.1.3.1 Mixed Function Oxidase

A mixed function oxidase is more accurately described as an enzyme complex consisting of three parts (Janig and Pfeil 1978, Figure 1-1). The first component, called cytochrome P_{450}, is responsible for binding molecular oxygen and the substrate. The name was derived from its ability to react with carbon monoxide and display a distinctive absorption maximum at 450 nanometers (Omura and Sato 1964). It also contains an NADPH-dependent cytochrome P_{450} oxidoreductase and an NADH-linked cytochrome b_5. These two enzymes, along with their cofactors NADPH and NADH, supply the reducing equivalents needed for oxidizing foreign and endogenous compounds (Hildebrandt and Eastabrook 1971). Finally, these enzymes are embedded in a phospholipid

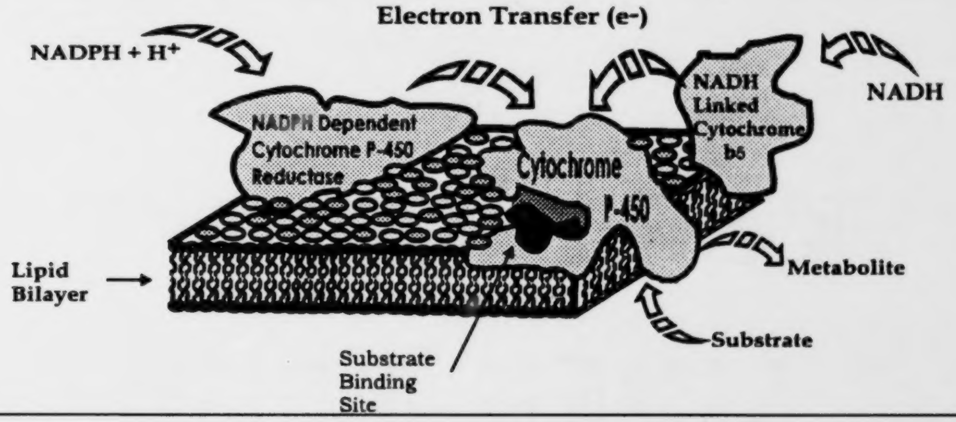

Electron Transfer (e-)
NADPH + H+
NADH
NADPH Dependent Cytochrome P-450 Reductase
Cytochrome P-450
NADH Linked Cytochrome b5
Lipid Bilayer
Metabolite
Substrate
Substrate Binding Site

Figure 1-1. Protein components of the mixed function oxidase embedded in the phospholipid bilayer of the endoplasmic reticulum.

bilayer which plays an important role in electron transfer, substrate binding, and alteration of the iron spin state (Vergeres *et al.* 1989). The requirement of a lipid component for activity is a principal reason this enzyme complex has been difficult to solubilize and study. Even though this enzyme is often described as a single entity, it is important to emphasize that it is comprised of three components functioning in concert. Overall, in the presence of a suitable substrate, cooperation within the enzyme complex yields water, NADP$^+$ and an oxidized metabolite.

1.1.3.2 Cytochrome P_{450}

Cytochrome P_{450} is an important part of the monooxygenase enzyme complex because of its ability to bind a substrate and molecular oxygen. Based on its dual roles the enzyme structure can be described by two components. The first is a heme prosthetic group (Sakurai *et al.* 1979), containing an iron porphyrin ring, called protoporphyrin IX (Figure 1-2). Ferric iron is the element responsible for binding molecular oxygen. The porphyrin ring is covalently attached by an axial thiolate ligation to a cysteine residue (Omuro and Sato 1964) located in the second, or protein part, of the enzyme (Figure 1-3). The protein component is responsible for coordinating the heme prosthetic group and accepting electrons. It also binds the substrate (drug) and orients it into the

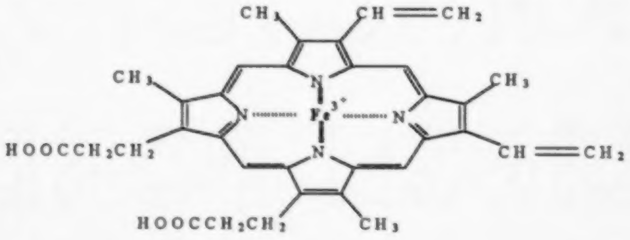


Figure 1-2. Structure of ferric protoporphyrin IX, the prosthetic group of cytochrome P_{450}.

active site (Koymans *et al.* 1993). This allows the enzyme to display stereoselectivity with chiral substrates (Trager 1989).

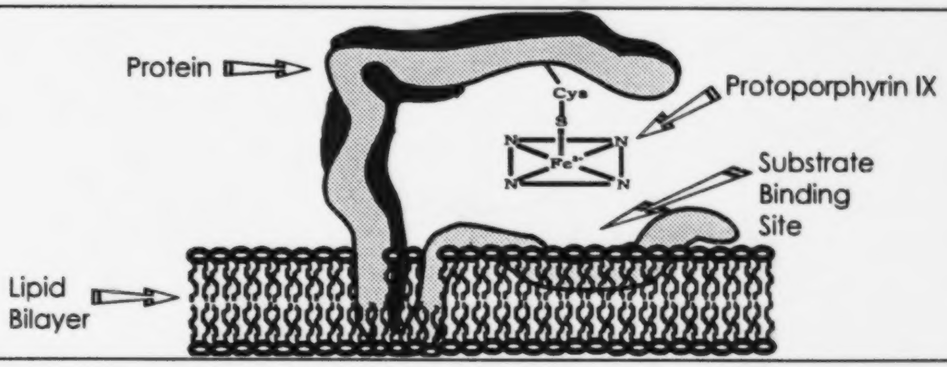


Figure 1-3. Simplified representation of heme iron coordination in relation to the substrate binding site in cytochrome P_{450}.

An overview of the cytochrome P_{450} structure is not complete without emphasizing that it is not a single enzyme but rather a family of closely related hemoproteins (Lu and West 1980). In general, they have a molecular weight ranging from 45,000 to 60,000 and can be found embedded in the smooth endoplasmic reticulum of most cells. As a group, they have an identical heme prosthetic group but differ in their amino acid composition and overall protein structure (Nebert and Gonzalez 1987). It is the existence of multiple forms of a single enzyme type that allows them to catalyze the oxidation of numerous compounds. This includes endogenous molecules like fatty acids, steroids, prostoglandins, leukotrienes, and biogenic amines as well as drugs, and environmental pollutants (Guengerich 1991). The enzyme has been detected in almost all tissues including the lung, kidney, brain, gastrointestinal tract, bladder, and blood platelets with the highest concentration being found in the liver (Black and Coon 1987). It is important to emphasize that these enzymes are not restricted to humans. They are found throughout the animal, plant

(Donaldson and Luster 1991) and fungal (Smith *et al.* 1993, Honeck *et al.* 1985, Wiseman 1980) kingdoms as well as in procaryotic cells like bacteria (Sligar and Murray 1986). A more detailed discussion of the cytochrome P_{450} enzyme system can be found in the following review articles: Guengerich 1993, Murray 1992, Porter and Coon 1991, Guengerich 1990, Gonzalez 1989.

With the advancements made in molecular biology, Nebert *et al.* (1987b) proposed a nomenclature system to classify this family of enzymes. Historically, isozymes were identified according to the reference compound they were found to metabolize. This method was sufficient until it was determined that independent labs were studying identical enzymes that were named differently. Therefore, a more reliable nomenclature was required. To avoid confusion, a system was proposed dividing the isozymes of cytochrome P_{450} into families, subfamilies, and individual enzymes. In addition, a distinction was made between the name used for the enzyme's gene and the protein. DNA corresponding to a cytochrome P_{450} gene was identified using the italicized root symbol *CYP*, an Arabic number designating the enzyme's family, a letter indicating the subfamily, and a numeral representing the individual gene (e.g. *CYP*2D6). An identical nonitalicized symbol was used when naming the protein component (e.g. CYP2D6). Cytochrome P450 protein with 40%, or greater, sequence identity are included in the same family and those with greater than 55% identity are included in the same subfamily. Using this nomenclature, 36 CYP protein families and 221 *CYP* genes have been identified in nature (Nelson *et al.* 1993). Of these families, CYP1, CYP2, CYP3, and CYP4 are the mammalian P_{450}'s involved in the oxidation of foreign compounds (Wrighton and Stevens 1992).

1.1.3.3 Catalytic Cycle of Cytochrome P_{450}

Isozymes of cytochrome P_{450} have the ability to oxidize many chemically distinct molecules using a cyclic series of chemical reactions (Figure 1-4).

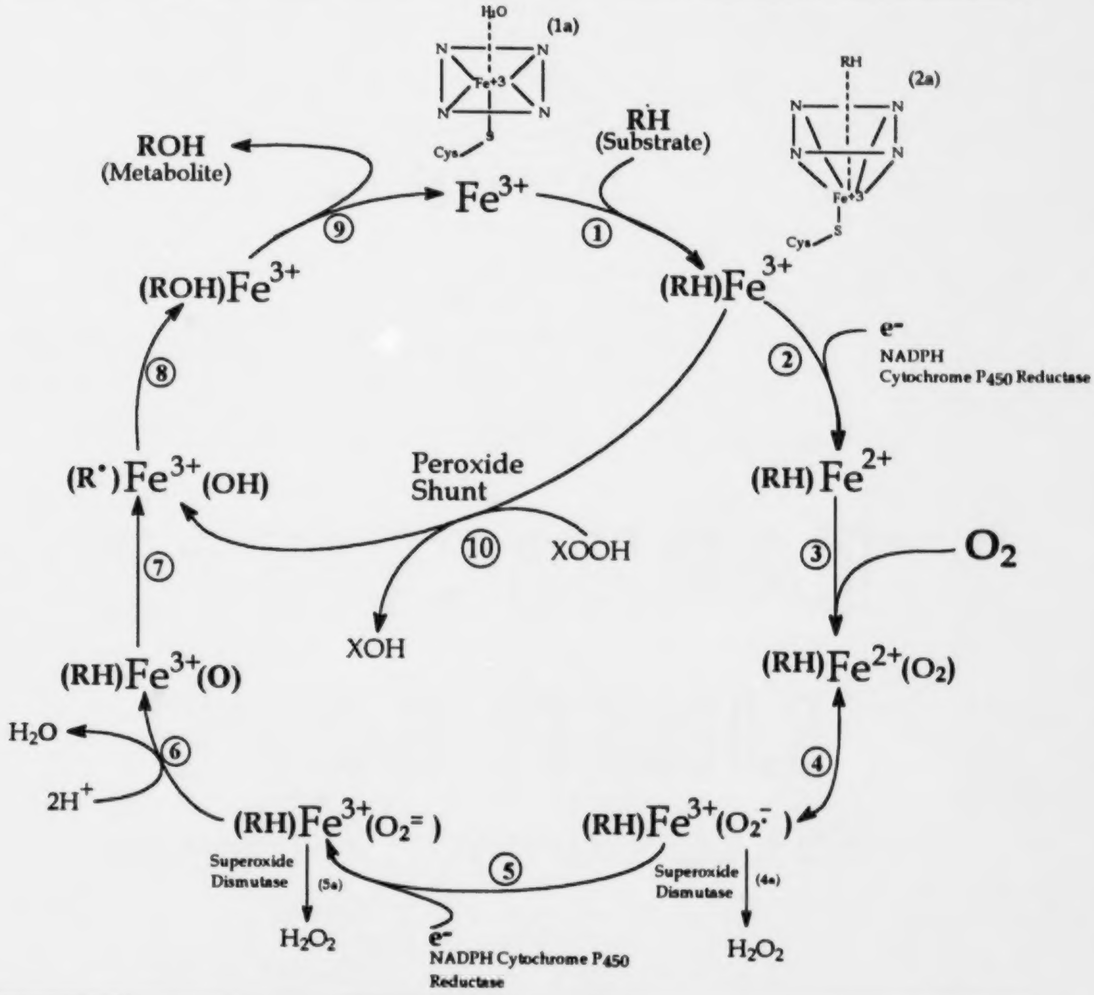

(1a)
(2a)
RH
(Substrate)
ROH
(Metabolite)
Fe3+
(ROH)Fe3+
(RH)Fe3+
e-
NADPH
Cytochrome P450 Reductase
(R•)Fe3+(OH)
Peroxide
Shunt
(RH) Fe2+
XOOH
XOH
O2
(RH)Fe3+(O)
(RH)Fe2+(O2)
H2O
2H+
(RH)Fe3+(O2=)
(RH)Fe3+(O2-)
Superoxide
Dismutase
(5a)
(4a)
H2O2
e-
NADPH Cytochrome P450
Reductase
①
②
③
④
⑤
⑥
⑦
⑧
⑨
⑩

Figure 1-4. A summary of the individual reactions comprising cytochrome P_{450}'s oxidative catalytic cycle (Coon *et al.* 1992).

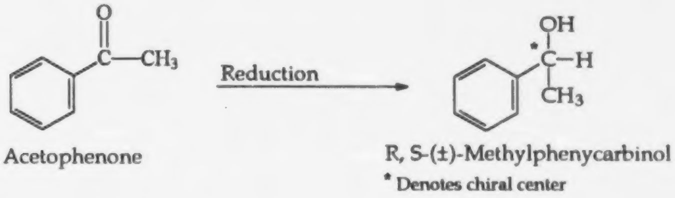
Oxidation begins when a substrate binds to the active site [substrate binding site (Figure 1-3)] of cytochrome P_{450} ①. This perturbs the spin state equilibrium

(Macdonald *et al.* 1989) of heme iron out of the nitrogen plane (1a and 2a) facilitating the uptake of an electron from NADPH cytochrome P_{450} oxidoreductase ②. This changes the heme group from ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron ③. Ferrous heme is the conformation capable of binding one molecule of molecular oxygen (O_2) forming a radical anion ④ (White and Coon 1980). A second electron, which is provided by cytochrome b_5, is transferred to oxygen creating a divalent anion ⑤. The reactive species of oxygen formed in steps ④ and ⑤ can dissociate from cytochrome P_{450} (Pompon 1987). If the cycle becomes uncoupled at this point, the enzyme superoxide dismutase can form hydrogen peroxide from the displaced oxygen anion (4a and 5a). Through a mechanism not well understood, the next step involves splitting the oxygen bond (Dawson 1988) followed by an uptake of two protons, resulting in the formation of an activated oxygen species and a molecule of water ⑥. Oxygen is then inserted by abstracting a hydrogen atom (Groves *et al.* 1978) from the substrate ⑦ and recombining the hydroxyl and carbon radicals ⑧ to yield a hydroxylated product. Finally, dissociation of the substrate from the active site ⑨ restores the hemoprotein to the ferric state and the cycle can begin again.

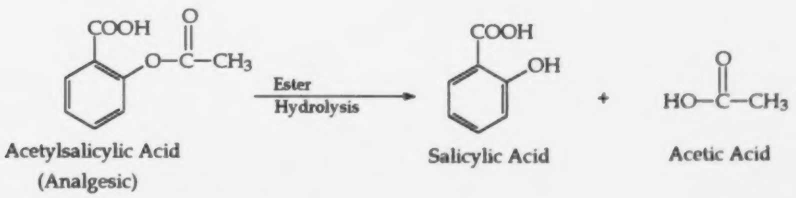
This catalytic cycle is capable of oxidizing a substrate without using molecular oxygen and NADPH + H^+ ⑩. It was discovered that plant and fungal cytochrome P_{450}'s are capable of using a peroxide (XOOH) to oxidize a substrate. The peroxide acts as an oxygen and electron donor. The overall reaction yields an oxidized product (ROH) and a molecule of water (HOH). This is known as the peroxide shunt (Hollenberg 1992, Blake and Coon 1981).

1.1.4 Reductive and Hydrolytic Drug Metabolism

In addition to oxidative biotransformations, other reactions play an important role in the metabolism of drugs and xenobiotics. One such reaction is the bioreduction of compounds containing nitro, carbonyl, and azo groups generating alcohol and amino derivatives respectively. Hydroxyl or amino groups on a drug will increase a molecule's polarity and enhance its susceptibility to conjugation reactions. An example of this is the reduction of the prochiral center in acetophenone to yield the chiral alcohol (±)-methylphenylcarbinol. The resulting metabolite is more polar than the parent molecule (Jenner and Testa 1973).


Reduction
Acetophenone
R, S-(±)-Methylphenycarbinol
* Denotes chiral center

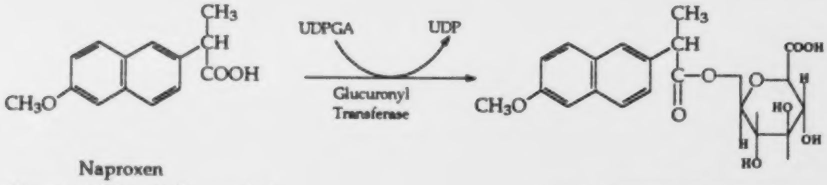
Hydrolytic enzymes present in tissues and plasma metabolize ester or amide linkages to their corresponding carboxylic acids, alcohols, phenols, and amines (Junge and Krisch 1975). These functionalities are generally more polar or reactive than the groups they were created from. The outcome and purpose of hydrolysis is similar to oxidative and reductive biotransformation. The drug or xenobiotic comes into contact with an enzyme system that alters its structure in order to enhance elimination or decrease toxicity. A classical example of a hydrolytic reaction is the conversion of the prodrug aspirin to salicylic acid (Hutt *et al.* 1986). An acetyl group is removed to uncover a phenolic group on the parent molecule. This illustration demonstrates that a metabolic reaction can activate a compound. Even though the metabolite, salicylic acid, is more polar than acetylsalicylic acid, it still possesses pharmacological activity.


Ester
Hydrolysis
Acetylsalicylic Acid
(Analgesic)
Salicylic Acid
Acetic Acid

1.1.5 Conjugation

Biotransformation of a drug *via* phase I metabolism, can result in detoxification, bioactivation, or toxicity. The outcome of these reactions varies because addition or generation of a new functional group is determined by the parent molecule's chemical structure. A mechanism the body utilizes to protect itself from reactive phase I metabolites is phase II, or the conjugation pathways of biotransformation. Conjugation enzymes attach polar, ionizable, endogenous molecules to reactive functional groups which can be found on, or introduced to, the parent compound (Krishna and Klotz 1994, Vree *et al.* 1992). Products of this reaction are highly water soluble, nontoxic, and generally lack pharmacological activity. Examples of endogenous molecules that are attached to foreign compounds include glucuronic acid, sulfate, glycine, and methyl or acetyl groups.

Glucuronic acid is the most commonly used endogenous molecule in phase II drug metabolism (Miners and Mackenzie 1991). A reason for this is that its precursor, D-glucose, is readily available in the body. In addition, the reactive form of glucose used for this reaction, uridine diphosphate glucuronic acid (UDPGA), can react with a large number of different functional groups. As with cytochrome P_{450} oxidations, this conjugation reaction offers flexibility. The β-glucuronide formed, after UDPGA is attached to an appropriate functional group on the xenobiotic, is generally less lipophilic than the parent molecule. In the majority of cases this will decrease toxicity and augment excretion into urine. An example of a drug that undergoes glucuronidation is naproxen (Mouehli and Schwenk 1991).



Naproxen
(Nonsteroidal antiinflammatory)

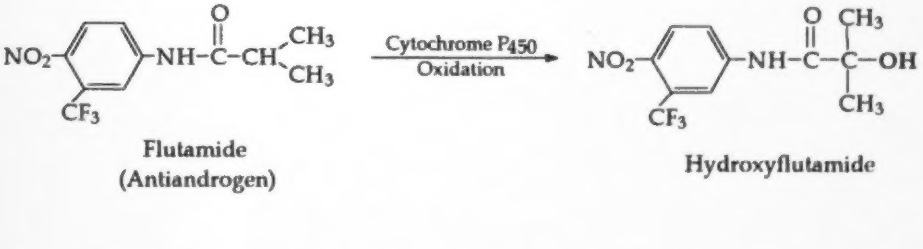
β-D-glucuronide of Naproxen

Glucuronidation is just one example of a phase II reaction that occurs in the body. Additional conjugation reactions are listed in table 1-1. Phase II reactions provide an effective defense against reactive phase I metabolites and the numerous chemical challenges the body is exposed to.

1.1.6 Formation of Metabolites With Increased Activity

The preceding examples were included to demonstrate how phase I and II reactions function to protect the body from the toxic effects of foreign compounds. An intruding molecule is chemically modified preventing accumulation in tissue where it can exert a deleterious effect. However, this is not the only consequence of biotransformation reactions. Metabolism can transform an "inactive" xenobiotic into active and toxic metabolites (Guengerich and Liebler 1985).

Flutamide is an example of a drug whose pharmacological activity is due in large part to an oxidative metabolite. It is used as a nonsteroidal antiandrogen to treat prostatic cancer (Brogden and Clissold 1989). When administered orally (250 mg dose), peak plasma concentrations of flutamide vary between 10 and 20 nanograms per milliliter (ng/mL). In contrast, the concentration of the hydroxylated metabolite, hydroxyflutamide, ranges between 1290 and 1320 ng/mL at the same time period. These data suggests that flutamide is rapidly metabolized to the hydroxy metabolite.

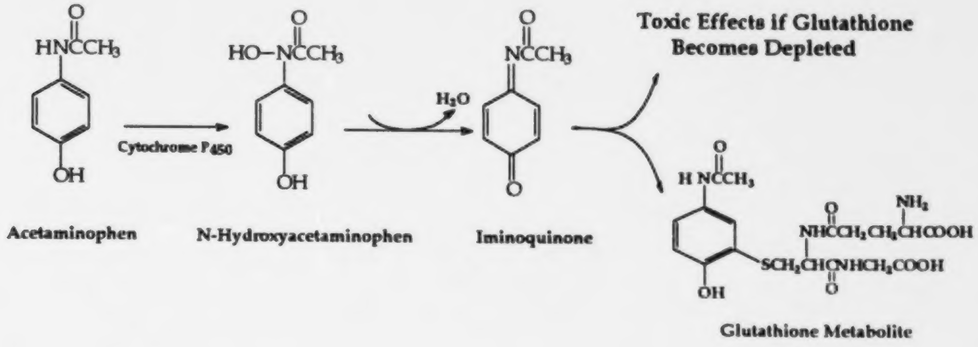


In animal studies, flutamide was shown to be a specific antagonist of androgen receptors. However, when tests were repeated using the oxidative metabolite, hydroxyflutamide, it was found to be more active than the parent drug (Berson *et al.* 1993). This example illustrates that adding a hydroxyl group, utilizing cytochrome P_{450}, creates a metabolite with increased activity. This information is essential for determining an effective way of delivering flutamide's therapeutic benefits. Using flutamide plasma concentrations alone to monitor activity could have dire consequences.

1.1.7 Formation of Reactive Metabolites

Products from metabolism have demonstrated an ability to cause adverse effects in the body. Generally, chemicals that elicit detrimental reactions are not usually toxic in themselves but require some sort of metabolic activation (Guengerich *et al.* 1990, Vamvakas and Anders 1990, Boyd and Statham 1983). This can be provided by phase I and II metabolism which can bioconvert parent compounds into electron deficient molecules called electrophiles. Many of these intermediates, some of which are formed by cytochrome P_{450}, are extremely reactive. By avoiding the normal detoxification enzymes, they attach to intracellular molecules including DNA and protein. Covalent modification of endogenous molecules will alter their activity leading to mutations, cell death, and possibly tumors (Guengerich 1992, Miles and Wolf 1991).

An example demonstrating how drugs can be converted into toxic products was the biotransformation of acetaminophen. Typically, this drug was excreted as a glucuronide or sulfate (Brodie and Axelrod 1949). N-hydroxyacetaminophen was a minor metabolite formed by cytochrome P_{450}. In the liver, this dehydrated to an iminoquinone which was inactivated by a reaction with glutathione. Under normal circumstances, this would be the metabolic fate of acetaminophen. However, when excessive amounts are ingested, and if glutathione stores become depleted, another scenario presents itself. The iminoquinone formed can react with cell constituents resulting in liver cell necrosis (Roberts *et al.* 1987). The outcome of this can be fatal (Corcoran *et al.* 1980, Davidson and Eastham 1966).


Toxic Effects if Glutathione Becomes Depleted
Cytochrome P450
H₂O
Acetaminophen
N-Hydroxyacetaminophen
Iminoquinone
Glutathione Metabolite

Another example of a molecule innocuous intact which, can become toxic after oxidative biotransformation, is aflatoxin b_1. This is a secondary metabolite and potential carcinogen secreted into the environment by the fungus *Aspergillus flavus*. It is a cause of considerable concern in many parts of the world because it can become a contaminant in stored grains (Jelinek *et al.* 1989).. Several lines of evidence suggest that conversion of the 8,9-double bond to the epoxide is an important step in tumor initiation (Figure 1-5). Evidence for genotoxicity

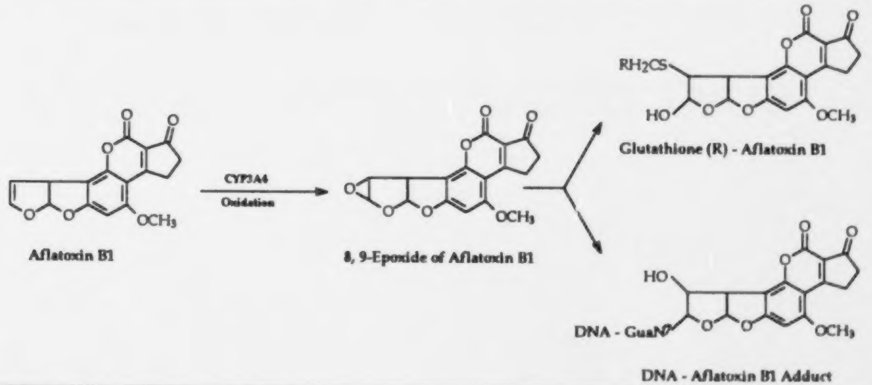

CYP3A4
Oxidation
Aflatoxin B1
8, 9-Epoxide of Aflatoxin B1
Glutathione (R) - Aflatoxin B1
DNA - Aflatoxin B1 Adduct

Figure 1-1. Conversion of Aflatoxin B_1, by cytochrome P_{450}, into a reactive epoxide that can covalently bind to DNA or glutathione.

arises from a DNA adduct which has been isolated and characterized form rat liver and called 8-(N^7-guanyl)-9-hydroxy-8,9-dihydroalflatoxin b_1. This can only be formed if DNA reacts with the 8,9-epoxide (Eaton and Gallagher 1994).

The preceding examples of phase I and II metabolism demonstrate that human biotransformation reactions have varied outcomes. In addition, if the drug of interest was chiral, the enantiomers can display differences in metabolism (Beckett 1990, Kaye 1990, Lennard *et al.* 1990). This makes it difficult to predict the activity or possible side effects of a molecule prior to it entering the body. Therefore, the metabolic fate of a drug must be determined experimentally using an appropriate modeling system before it can be approved for human use. The model determines the manner in which a xenobiotic is metabolized and whether the metabolites possess desirable activity or toxicity.

1.2 *In vitro* Models of Mammalian Metabolism

For ethical reasons, humans are not used in preliminary drug metabolism studies. In addition to the possibility of toxic side effects, their outcome is difficult to predict. For this reason other species of mammals are used. Much of the knowledge gained, regarding the manner in which drugs are metabolized, has been obtained from animal modeling systems. Testing is based on the premise that the organism chosen will provide information that can be used to predict human metabolism. This is achieved by isolating, separating, and identifying the parent drug, as well as its metabolites, from the modeling system. However, an animal model that in all cases accurately reflects human *in vivo* metabolism does not exist. Therefore, studies are conducted on a standard group of test animals in order to piece together a metabolic profile that can accurately predict the safety of a drug prior to human consumption.

Use of laboratory animals, to model human metabolic reactions, is an accepted and well established method for testing new chemotherapeutic agents.

However, there are drawbacks to using animals for testing the safety of products prior to human use. For example, the cost of obtaining and caring for laboratory animals is large. There is the expense of special facilities for lodging and a trained staff for their care. In addition, the general public has an unfavorable perception of the need to use animals for testing. Subsequently, efforts are ongoing to decrease their use.

Finally, the quantity of phase I metabolites recovered from laboratory animals is quite small. In addition, conjugation can reduce this amount even further. If large amounts of a metabolite are required for additional pharmacological evaluation, increasing the dose to improve yield will not improve this situation. It will likely alter the metabolic profile or, in some cases, be lethal. If a metabolite is of clinical significance and difficult to synthesize chemically, a large number of animals will be sacrificed to obtain quantities sufficient for meaningful analysis. It would seem logical and advantageous to investigate alternative models to assist preliminary drug metabolism studies.

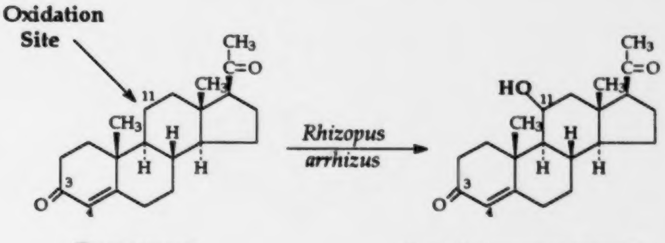
A rapidly growing trend in the pharmaceutical sciences is the application of *in vitro* models to reduce, replace, or decrease the use of laboratory animals for drug testing (Birkett *et al.* 1993, Wrighton *et al.* 1993). The stimulus for this change in direction has come from advancements in cellular and molecular biology (Goldberg *et al.* 1993, Remmel and Burchell 1993). *In vitro* tests are used, in the same manner as animal models, to predict if a molecule can have adverse effects on living beings. This information is used to judge whether a drug is safe for consumption. A partial list of the new techniques involved would include the use of isolated human organs, tissues, and subcellular fractions (microsomes) whose cytochrome P_{450} content has been characterized (Raucy and Lasker 1991, Ryan and Levin 1990). Isolated human enzymes are used to determine the manner in which a compound is metabolized *in vivo*. In addition, if monoclonal antibodies are used, it is possible to ascertain which specific enzymes are involved (Gelboin 1993). With advancements being made in mammalian cell culture techniques, it is now possible to propagate human liver hepatocytes that retain their *in vivo* activity (Li *et al.* 1991, Fabre *et al.* 1990). Therefore, the whole cell can be used for metabolic studies. Another approach is to use individual or groups of cytochrome P_{450} enzymes that were cloned from human cDNA and expressed in an appropriate cell (Beaune *et al.* 1993, Langenbach *et al.* 1992). A range of cell lines containing specific cytochrome P_{450} enzyme(s) can be used to determine if an enzyme is capable of metabolizing a particular drug. This will point the way to a likely pathway of metabolism in humans. This technique, in conjunction with *Salmonella typhimurium* reversion tests, can be used to determine whether a metabolite is a potential carcinogen (Ames *et al.* 1973). A futuristic extension of this approach is to use multicellular transgenic organisms, which possess minimal endogenous cytochrome P_{450} activity, to generate an *in vivo* metabolic profile (Jowett *et al.* 1991). By using specific markers, these organisms will help identify toxic or carcinogenic metabolites. Finally, there is one more *in vitro* technique that can be used to study human drug metabolism. It is the application of microorganisms. The scientific literature contains many reports of microbial transformations that can mimic the biotransformation reactions occurring in mammals (Griffiths *et al.* 1991, Davis 1988, Clark *et al.* 1985, Rosazza and Smith 1979).

1.2.1 Microbial Models of Mammalian Metabolism

1.2.1.1 Introduction

Using microorganisms to carry out transformations has been observed throughout history. Many traditional processes like leavening of bread, making beer, wine, and cheese all require the action of microorganisms. In the early 1900's, the economic potential of harnessing this ability was being recognized. Microorganisms were used industrially to modify or produce many different products by simple and well defined reactions (Sebek 1982). A list of these products would include ethyl alcohol, acetone, butanol, and citric acid. However, it was the large scale production of penicillin that created a flurry of research into the applicability of industrial microbiology (van der Beek and Roels 1984). Attention was focused on the bioenergetic pathways the cell used to synthesize endogenous molecules and how these could be exploited to improve yield. This was followed by numerous reports identifying new microorganisms that could be used to generate unique compounds with biological activity.

An important discovery, that increased the awareness of this type of work, was made by Peterson and Murray in 1952. They found a fungus, *Rhizopus arrhizus*, that was capable of biotransforming progesterone to 11-α-hydroxyprogesterone. The significance of this finding was two fold. First, a fungus was utilized to catalyze on an industrial scale a specific reaction that was extremely difficult to reproduce chemically. Secondly, a chemical reaction could be used to convert 11-α-hydroxyprogesterone to cortisone which was important to anyone suffering from arthritis. Cortisone was found to have powerful antiinflammatory properties making it an ideal treatment for this condition. The outcome of moving this fermentation from the bench top to an industrial scale decreased the price of cortisone from approximately two hundred dollars per treatment, in 1950, to less than one dollar per gram of compound by the mid 1980's (Smith *et al.* 1993). The success of this biotransformation initiated extensive screening efforts that focused on isolating microbes with similar



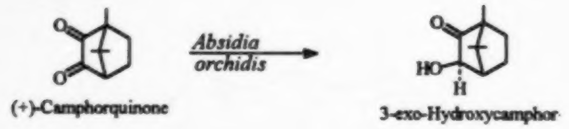
Progesterone

11-α-Hydroxyprogesterone

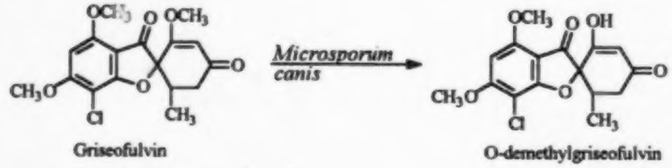
activity. This effort produced catalogs of information on specific types of biotransformations (Beukers *et al.* 1972). A few of the reactions types uncovered included oxidation, reduction, dealkylation, and hydrolysis (Table 1-3).

Table 1-3. Specific examples of microbial biotransformation reactions (Beukers *et al.* 1972).

Reduction



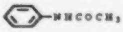
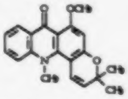
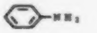
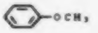
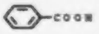
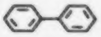
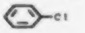
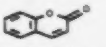
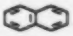
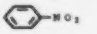
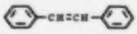
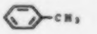
Dealkylation



This body of information was applied to mammalian drug metabolism and reviewed in 1974 when Robert V. Smith and John P. Rosazza published a paper entitled, "Microbial Models of Mammalian Metabolism". Smith and Rosazza wanted to demonstrate that microbial biotransformations could be used to model human metabolic reactions. In order to test the feasibility of creating a microbial modeling system they chose to concentrate on a single type of mammalian phase I reaction. They focused on aromatic hydroxylation in order to compare their experimental results with known mammalian biotransformations. Thirteen aromatic compounds were chosen whose hydroxylation by mammals had been well documented. This would simplify the task of making comparisons between the two biotransformation systems (Table 1-4).

This experiment demonstrated that a microbial system will generate results that are in correlation with reported mammalian *in vivo* and *in vitro* metabolic profiles. Therefore, Smith and Rosazza knew it was possible to establish a microbial model, consisting of a selected group of microorganisms, that could mimic mammalian metabolism. The selected microbes could be used to generate a metabolic profile in parallel with mammalian studies. One advantage to using microorganisms in this manner is that if a metabolite is common to both systems, microbial metabolite production can be scaled up.

Table 1-4. A list of the thirteen compounds chosen to test the feasibility of microbial models (Smith and Rosazza 1974).

Substrate	**Microbial Metabolites**	**Mammalian Metabolites** (*in vitro*)	**Mammalian Metabolites** (*in vivo*)
Acetanilide 	2-OH-Acetanilide, aniline	2-OH-Acetanilide, aniline	2-OH-Acetanilide, aniline
Acronycine 	9-OH , 11-OH , 9, 11-$(OH)_2$ acronycine	Not Reported	9-OH , 11-OH , 9, 11-$(OH)_2$ Acronycine
Aniline 	Acetanilide, 2-OH-acetanilide, 4-OH- aniline	4-OH- aniline	Acetanilide, 2, 3, and 4- OH aniline
Anisole 	2 and 4-OH -Anisole, phenol	2 and 4- OH-Anisole, phenol	2 and 4- OH -Anisole
Benzene 	Phenol	Phenol	Phenol
Benzoic Acid 	2 and 4-OH-Benzoic Acid, 3, 4-$(OH)_2$- Benzoic Acid	3-OH-Benzoic Acid	2, 3, and 4-OH-Benzoic Acid
Biphenyl 	2 and 4-OH-Biphenyl 4, 4-$(OH)_2$-Biphenyl	2 and 4-OH-Biphenyl	4-OH, 3, 4, $(OH)_2$ 4, 4'-$(OH)_2$-Biphenyl
Chlorobenzene 	2 and 4-OH - Chlorobenzene	2, 3, and 4-OH Chlorobenzene	2, 3, & 4-OH-Chlorobenzene
Coumarin 	7-OH - Coumarin	7-OH - Coumarin	3 and 7-OH - Coumarin, (4, 5, 6, and 8-OH) - Coumarin
Naphthalene 	1 and 2-OH - Naphthalene	1 and 2-OH - Naphthalene	1 and 2-OH - Naphthalene
Nitrobenzene 	————	4-OH - Nitrobenzene	2, 3, and 4-OH-Nitrobenzene, 2, 3, and 4-OH-Aniline
trans-Stilbene 	4-OH, 4, 4', $(OH)_2$ - trans-Stilbene	4-OH, 4, 4', $(OH)_2$ - trans-Stilbene	4-OH, 3, 4, $(OH)_2$ and 4, 4', $(OH)_2$ trans-Stilbene
Toluene 	2 and 4-OH- Toluene	2 and 4-OH- Toluene, benzyl alcohol	Benzoic acid and conjugates

Suitable quantities of a given metabolite could also be available for structure elucidation and determination of pharmacological activity.

Labeled compounds may be used to augment the information obtained regarding the manner in which xenobiotics are metabolized. When new compounds are investigated for pharmacological activity, an analytical method may not be available to measure the parent drug in biological samples. Even though it is possible to speculate on the potential metabolites, detecting them can be extremely difficult. However, if a label can be attached to the parent molecule, a number of versatile analytical methods could be employed to determine the fate of a drug and its metabolites (Hawkins 1988). Specific techniques would include utilization of autoradiography (Rogers 1979) and liquid scintillation counting (L'Annunziata 1987). An important advantage of these techniques was the specificity and sensitivity of detection achieved if the label was in a stable position. This technique can be exploited by microorganisms to predict and identify possible human metabolites. If an isotopically labeled drug is used as the substrate, semi-preparative amounts of isotopically labeled metabolite can be produced and detected in biological systems using the techniques listed.

Use of microorganisms in parallel with mammalian models has additional advantages including a decreased demand for laboratory animals. This is significant due to the increased vigilance displayed by animal rights groups. Microbial systems are substantively cheaper to purchase than animals and, in addition, they do not require costly, dedicated holding facilities or the same level of care. Further, the problem of detecting nascent, highly reactive metabolites can be addressed using microorganisms because they can tolerate higher concentrations of drug. In addition, microorganisms carry out biotransformations under mild reaction conditions that are often regiospecific and stereospecific. Finally, microbial biotransformation techniques are easy to learn and the equipment required is relatively inexpensive (Goodhue 1982). This is an important consideration when microbial transformations are being compared to chemical synthesis or other *in vitro* techniques for the production of a metabolite.

1.2.1.2 How Fungi are Used To Model Mammalian Metabolism

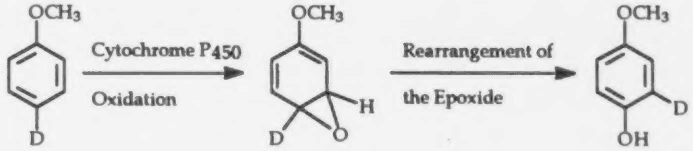
Using a microbial biotransformation system provides the type of flexibility that cannot be found with laboratory animals. When a specific metabolite of a drug is required or if a range of metabolites is desired, simple screening procedures can often be used to generate them (Davis 1988). Instead of choosing a mouse, rat, cat, or dog, a microorganism is selected based on its metabolizing capabilities and the product(s) desired. The drug is then added to a flask containing the organism of choice and after a short incubation time, the growth media is tested for the appearance of the desired metabolites. If the initial screen is successful, scale-up of the most productive microbes can be started to biosynthesize semi-preparative amounts of metabolite for additional evaluations.

To carry out an initial screening process, several fungi are selected in order to test their ability to metabolize a drug substrate (Table 2-2). Bacteria can also be used for biotransformation experiments (Cork and Krueger 1991) though they tend to metabolize foreign, aromatic substances with dioxygenase enzymes to form catechols (Haigler and Spain 1991). These are further oxidized to carbon dioxide and water which does not provide the type of information required. The selection of specific microbes will be based on experience, intuition and literature precedents since there is a wealth of accumulated knowledge in this area. Once the organisms are selected a two stage fermentation process is generally employed. Stage I uses a submerged culture system, with 125 mL Erlenmeyer flasks, where the microbes are grown on 25 mL of culture medium (e.g. yeast extract/glucose media). This proceeds for approximately 72 hours to achieve optimal cell growth and maximal cell density. Stage II cultures are initiated from the biomass generated in stage I in order to decrease the lag phase of growth. It is at this point that substrate is added dissolved in a non toxic solvent like water, methanol, ethanol or DMSO (if substance is not water soluble). After the drug has been added, the culture is allowed to incubate and scheduled broth samples are taken to test for the appearance of metabolites. If a microbe is found that can metabolize a drug, the procedure can be scaled up into larger production vessels.

When utilizing a two stage screening process it is extremely important to use the same standard procedures for each microbe tested. There are many factors which can influence the type of metabolites produced. The most important factors are media composition, aeration, pH, temperature and substrate concentration. If these conditions are constant for phase I and II growth, the process of scale-up can be simplified by optimizing one factor at a time. Once the procedure has been optimized it becomes possible to produce "unlimited" amounts of a specific metabolite.

1.2.1.3 Microbial Oxidative Metabolism

An important question that has not been addressed is the reason for using microorganisms as a model for mammalian metabolism. Part of the answer can be found in the similarity between their cell types. Mammals and fungi are comprised of cells that are termed eucaryotic. Therefore, in general, they will both have a similar subcellular architecture containing a nucleus, mitochondria, endoplasmic reticulum, and other organelles. They also possess similar enzyme systems. For example, fungi have the ability to oxidize compounds using a hemoprotein containing a cytochrome P_{450} system which is similar to the one found in mammalian microsomes. Fungal and mammalian cytochrome P_{450} systems are generally water insoluble, require NADPH for activity, possess a flavoprotein oxidoreductase and a heme containing protein (Smith *et al.* 1993, Honeck *et al.* 1985). In addition, Auret *et al.* (1971) demonstrated that the fungus *Cunninghamella bainieri* oxidises benzene through an epoxide intermediate. This mechanism was originally elucidated using mammalian microsomes and is known as the NIH shift.



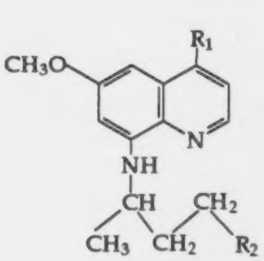
The major difference between mammalian and microbial enzyme systems is that the total cellular activities of a microorganism can lead to complete breakdown (metabolism) of a substrate. This can provide the organism with energy and celluar building blocks (Witholt *et al.* 1990). The cytochrome P_{450} associated with mammalian microsomes has a more specialized role in endogenous molecule oxidation and xenobiotic metabolism. In addition, fungal and mammalian enzymes are intracellular but the cell envelope surrounding them is different. Fungi have a rigid cell wall protecting them from the environment. Therefore, suitable nonspecific transport mechanisms must exist to move substrates through the cell wall allowing fungi to adapt to their surroundings. Transport systems also exist in the liver but are specialized to carry out specific duties of a particular cell. When fungi are used to perform biotransformation reactions, they usually do not obtain a direct benefit. This forces them to utilize reserve materials or medium components to replace the energy loss. The liver evolved as a specialized organ to carry out specific tasks supported by the rest of the body.

Finally, fungal organisms tend to have broad substrate oxidizing abilities making them similar in activity to hepatic cytochrome P_{450}. Specifically, members of the *Cunninghamella* genus of fungi show a high degree of parallelism with liver microsomal oxidations. They have demonstrated an ability to oxidatively dealkylate and N-demethylate various compounds and form phase II conjugates. What has yet to be determined about fungal biotransformations is the number of cytochrome P_{450} enzymes responsible for these reactions and whether the proteins and DNA involved are similar to the human system.

1.2.1.3.1 Using Microbial Models To Predict Oxidative Mammalian Metabolites

To demonstrate that fungi can be used to form oxidative metabolites, a study was conducted by Clark *et al* (1986) to predict a human metabolite for the antimalarial drug, 4-methylprimaquine [(I) 4-MPQ]. This compound was chosen because previous studies demonstrated that 4-MPQ possesses curative activity superior to the parent antimalarial compound primaquine (II). Because the metabolism of this compound was unknown, microbial studies were used in an effort to isolate, identify and predict probable mammalian metabolites. Previous microbial metabolism studies of the parent drug had shown that the major fungal metabolite isolated was later proven to be the major metabolite found in the plasma of rat, monkey and man.

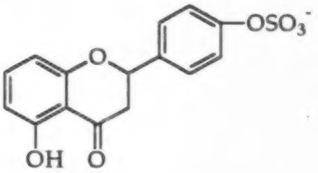
For preliminary screening, Clark chose selected strains of *Aspergillus* species and found that several of these were capable of metabolizing 4-MPQ, but only one metabolite was formed. Since *Aspergillus ochraceus* provided the greatest efficiency in conversion to this metabolite it was used in the scale-up procedure. This approach allowed Clark to identify the major metabolite as structure (III). From this information they predicted that III would be the major metabolite when this drug was given to man.



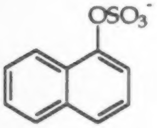
4-Methylprimaquine	I	$R_1 = CH_3$	$R_2 = CH_2NH_2$
Primaquine	II	$R_1 = H$	$R_2 = CH_2NH_2$
Metabolite	III	$R_1 = CH_3$	$R_2 = CO_2H$

1.2.1.4 Fungal Conjugation

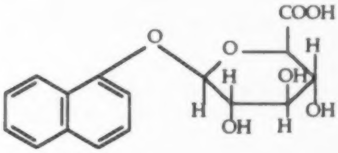
Conjugation reactions in mammals are an important part of the metabolic process responsible for removing toxic compounds. Even though these reactions are not as common in microorganisms, there are examples of fungi that can biosynthesize phase II metabolites. Ibrahim *et al.* (1989) demonstrated that the microorganism, *Streptomyces fulvissimus,* was able to biosynthesize a sulfate metabolite from the parent compound 5-hydroxyflavone. Another report by Cerniglia *et al.* (1982) found the fungus, *Cunninghamella elegans,* could form sulfate and glucuronide conjugates of the naphthol metabolite of naphthalene. 1-Naphthol, a phase I metabolite produced by *C. elegans,* has been shown to possess toxic properties. Therefore, conjugation with sulfate and glucuronic acid suggests these reactions might be important to detoxifying xenobiotics in fungi. They could also play an important role in neutralizing compounds having been activated by phase I metabolism.



4', 5-dihydroxyflavanone-4'-sulfate



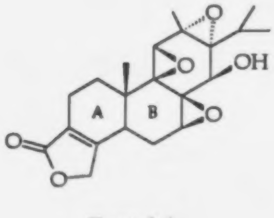
1-Naphthol Sulfate



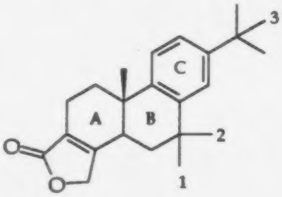
1-Naphthol Glucuronide

1.2.1.5 Formation of Metabolites With Altered Activity

The preceding sections demonstrated how microorganisms can be used in parallel with mammalian models to generate a metabolic profile of the possible oxidative phase I and some phase II metabolites. However, this is not the only application of a microbial biotransformation reaction. Metabolism can be used to change the activity of a xenobiotic. An example of this is the use of the fungus, *Syncephalastrum racemosum,* to hydroxylate triepoxide diterpenes that were isolated from the perennial herb, *Tripterygium wilfordii.* These compounds have shown promising antileukemic and antiinflammatory activity but their toxicity has limited their clinical usefulness. Milanova and Moore (1993) postulated that *S. racemosum* could be used to generate oxidative metabolites of a lactone analog of the parent compound. The intent was to isolate a metabolite that was clinically useful without the toxic side effects. Previous work determined that hydroxylation of the B - C ring system was the minimal requirement for pharmacological activity. Milanova was able to isolate and positively identify four oxidative metabolites whose production will be scaled up. They hope to biosynthesize enough compound for pharmacological testing.



Triptolide
(Triepoxide Diterpine)



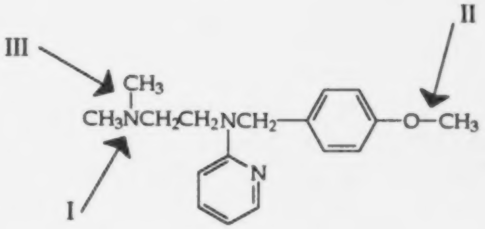
Analog of Triptolide

Oxidative Metabolites Produced by *R. racemosum*

	1	2	3
Metabolite #1	OH	H	H
Metabolite #2	H	OH	H
Metabolite #3	H	H	OH
Metabolite #4	H	=O	H

1.2.1.6 Formation of Reactive Metabolites

Microorganisms can also be used to study the production of reactive metabolites. Hansen *et al.* (1986) studied whether microorganisms could be used to biotransform the antihistamine pyrilamine maleate which is included in a variety of cold, allergy and sleep aid formulations. When this compound was tested in mammalian cell culture systems, it was discovered that pyrilamine can cause a concentration dependent increase in unscheduled DNA synthesis. This genotoxic potential initiated a series of studies which were designed to determine how it was metabolized and whether a metabolite was the problem. Using Smith and Rosazza's screening procedure, they found fourteen fungi capable of metabolizing pyrilamine. After further screening, two *Cunninghamella elegan* strains were chosen to continue testing because they produced the largest amount of metabolite. After optimization and scale-up, they were able to show that three major routes of fungal metabolism are involved in the biotransformation of this drug. One involves oxidation of the tertiary amine (I) while the other two are formed by oxygen (II) and nitrogen (III) demethylation.



Pyrilamine

It was discovered that these products complemented the three common mammalian metabolic reactions. When the genotoxic potential of these metabolites was tested (using reversion of *Salmonella typhimurium*) there was an absence of mutagenic activity. Even though this test proved negative, it is still possible to isolate some of the minor metabolites produced by other fungi to determine if one of them is the toxic metabolite.

1.3 Comparison of *in vitro* Metabolic Techniques Using Warfarin as a Probe

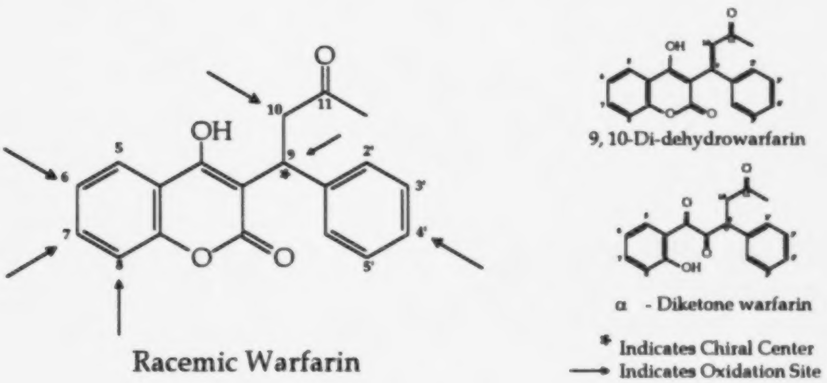
There are numerous *in vitro* techniques available for studying the manner in which a drug is metabolized. A question that arises, when examining these methods, is which one provides results that approximate the metabolic fate of a drug in humans. This is a difficult question to answer because if you examine data obtained from a small group of volunteers who were tested for their ability to metabolize a drug, it would be difficult to find an individual that would be considered representative. Therefore, a drug's metabolic profile is based on studies employing a large population. It is for this reason that development of a single metabolic model to represent humankind is extremely difficult. An alternative approach would be to use a range of techniques that provide complementary and overlapping information. An example of a drug that has been extensively used to study the various *in vitro* metabolic techniques is warfarin (Kaminsky 1989). Its use as a metabolic probe will be discussed to highlight the strengths and weaknesses of the *in vitro* methods that have been used to study its metabolism.

1.3.1 Warfarin Metabolism in Man

Racemic warfarin, a synthetic coumarin, is a vitamin K_1 antagonist (O'Reilly 1970) whose enantiomers display different potency (the S-(-) enantiomer is 6x more potent than the R-(+) enantiomer, Hewick and McEwen 1973). Clinically, it is used for the treatment of coagulation disorders such as thrombophlebitis, pulmonary embolism, and myocardial infarctions. It was chosen as a model substrate to study biotransformation reactions, because in humans it undergoes extensive stereoselective metabolism to various oxidized and reduced products. In addition, these products are not biosynthesized from a single type of cytochrome P_{450}. It was originally postulated that the concentration of the individual warfarin metabolites could be used to quantify specific microsomal cytochrome P_{450} levels (Kaminsky *et al*, 1980). There is also a difference in the way the enantiomers are metabolized. Finally, warfarin has a narrow therapeutic index (Barry and Feely 1990) and inhibition of its metabolism in humans is known to be the cause of several potentially dangerous interactions (Toon *et al*. 1986, Lewis *et al*. 1974).

An overview of the metabolic fate of R-(+) and S-(-)-warfarin ($t_{1/2}$ = 47.1 hours) in humans shows that it is extensively metabolized with very little intact drug being excreted in urine and feces (Lewis and Trager 1970). The metabolic fate of racemic warfarin is summarized in figure 1-6.

Figure 1-6. Human metabolism of racemic warfarin.


OH
O
O
O
5
6
7
8
9
10
11
2'
3'
4'
5'
Racemic Warfarin
OH
O
O
9, 10-Di-dehydrowarfarin
O
O
O
OH
α - Diketone warfarin
* Indicates Chiral Center
Indicates Oxidation Site

rac-Warfarin Metabolism			
Oxidative		Reductive	
Aromatic	Aliphatic	Aromatic	Aliphatic
6-Hydroxywarfarin	9-Hydroxywarfarin [1]	ND	11-Alcoholwarfarin [3]
7--Hydroxywarfarin	10-Hydroxywarfarin [1]		→ Warfarin alcohol-$_1$
8--Hydroxywarfarin			→ Warfarin alcohol-$_2$
4'--Hydroxywarfarin			
α-diketone warfarin [2]			

ND - none detected.

1 - These alcohols undergo dehydration, forming a double bond between C_9 and C_{10}.

2 - Warfarin undergoes oxidative ring cleavage to yield the α-diketone.

3 - Reduction of the carbonyl at C_{11} yields the diasterisomeric alcohols indicated with the subscripts 1 and 2.

A closer examination of the manner in which the enantiomers are metabolized reveals that the major metabolite for S-warfarin is different from R-warfarin (Table 1-5, Toon *et al.* 1986). S-warfarin is primarily oxidized to 7-hydroxywarfarin with a small amount being reduced to warfarin alcohol-$_2$. The major metabolite for R-warfarin is warfarin alcohol-$_1$. In addition, both enantiomers are oxidized to 6-hydroxywarfarin in equal amounts and small amounts of the other metabolites can be found in urine and feces. With the exception of 4′-hydroxywarfarin and the warfarin alcohols, which possess diminished activity, none of these metabolites is pharmacologically active. Therefore, with the knowledge of how warfarin is metabolized *in vivo*, a foundation exists by which a comparison of the *in vitro* techniques that have been used to study warfarin metabolism can be made. The present discussion focuses on methods that do not require the use of laboratory animals.

Table 1-5. Percentage of the total warfarin dose excreted in the urine and feces as metabolites or unchanged drug during a C^{14} study in a single patient.

	R-Warfarin (% radioactivity)		S-Warfarin (% radioactivity)	
	Urine	Feces	Urine	Feces
Warfarin	1.0	7.0	0.9	4.5
6-Hydroxywarfarin	9.4	1.0	8.1	0.62
7- Hydroxywarfarin	4.6	0.72	32.4	1.3
8- Hydroxywarfarin	7.0	0.31	0.14	ND
Warfarin Alcohol-1	8.1	0.82	ND	ND
Warfarin Alcohol-2	0.01	ND	1.3	ND

ND = not determined.

1.3.2 Use of Human Liver Microsomes to Study Warfarin Metabolism.

A technique that is widely used to model mammalian metabolism is the establishment of human liver banks to utilize human liver microsomes. Since one of the goals of drug metabolism studies is to determine the metabolic fate of a drug *in vivo*, using human tissue would be one way to estimate this *in vitro*. Kaminsky *et al.* (1984) evaluated warfarin metabolism with 33 different posrmortem human microsomal preparations generated from 33 human livers. Using this technique the following results were generated (Table 1-6):

Table 1-6. Mean rate of formation of the metabolites from R and S warfarin catalyzed by hepatic microsomal preparations from human subjects.

	Rate of Formation (nmol product / nmol P450 / min)	
	R-warfarin	S-warfarin
Dehydrowarfarin	22 ± 17	29 ± 24
6-Hydroxywarfarin	111 ± 90	38 ± 20
7-Hydroxywarfarin	44 ± 30	113 ± 73
8-Hydroxywarfarin	46 ± 34	1 ± 3
4′-Hydroxywarfarin	17 ± 10	32 ± 16
10-Hydroxywarfarin	40 ± 26	9 ± 8

As indicated by the data, this technique can provide a metabolic profile of the possible oxidative and reductive metabolites produced *in vivo*. However, as indicated by the standard deviations, there is a large difference between microsomal preparations as to the pathways utilized to metabolize warfarin. This is reflective of the *in vivo* situation in patients. The most common profile after considering all the data was one showing 6-R and 7-S-hydroxywarfarin as the most abundant metabolites. Another study conducted by Rettie *et al.* (1989) was undertaken to correlate *in vivo* data, obtained from six healthy volunteers, with *in vitro* data. The results they obtained (Table 1-7) using microsomal preparations from eleven whole postmortem human livers is similar to data from the previous study. The major difference is that in this study 4′-

hydroxywarfarin is the most abundant metabolite produced from S-warfarin. They speculate the reason for this was the concentration of warfarin used (0.2 ug/mL) was not reflective of the concentration that occurs *in vivo* (5-10 ug/mL). When a higher concentration of S-warfarin was used, they found that 7-hydroxywarfarin was the most abundant metabolite produced.

Table 1-7. Production of phenolic metabolites, using R and S-warfarin as the substrate, by human liver microsomes.

S-warfarin	MOST ABUNDANT	**R-warfarin**
4'-Hydroxywarfarin [5]		**6-Hydroxywarfarin** *
6-Hydroxywarfarin *		**8-Hydroxywarfarin** *
7-Hydroxywarfarin [1]	⇩	**7-Hydroxywarfarin** *
8-Hydroxywarfarin *	LEAST ABUNDANT	**4'-Hydroxywarfarin** *

* **Data correlates with previous study**

☐ **Order in study found in table 1-6**

The data from both of these studies demonstrates that the results are reflective of what happens *in vivo*. However, there is a significant amount of variability between microsome preparations[1]. This could be due to differing concentrations of cytochrome P_{450}, different concentrations of NADPH oxidoreductase, and altered microsomal membrane influences. Therefore, results from human liver microsomal preparations can be as variable as those obtained from human volunteers.

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[1] A method being used to deal with microsomal variability is to characterize the activity of certain cytochrome P_{450} enzymes for each liver used for study. This will generate a fingerprint of the overall activity you can expect from a microsomal preparation.

A practical drawback to using human liver microsomes to generate a metabolic profile is having the facilities to work with human tissue. This is a specialized highly regulated technique that would be expensive and impractical for the average research lab. In addition, obtaining large enough quantities of a metabolite for structure elucidation and pharmacological evaluation would be difficult.

1.3.3 Microbial Metabolism of Warfarin.

A technique that can provide similar information and overcome some of these drawbacks is the use of microbial models. Wong and Davis (1989), used the metabolic probe warfarin and a three stage fermentation process employing the fungus *Cunninghamella elegans* (ATCC 36122), to determine whether a similar metabolic profile to humans could be generated. They grew C. *elegans* in two stages for a total of 120 hours and inoculated the biomass (third stage), which was washed and resuspended in phosphate buffer, with 0.3 mg/mL of racemic warfarin. After 48 hours, they examined the phosphate buffer and discovered the fungus was able to generate a metabolic profile (Table 1-8) that was both

Table 1-8. Concentration (ng/mL) of oxidative metabolites biosynthesized by C. *elegans* after 48 hours in phosphate buffer.

	R-warfarin (ng/mL)	S-warfarin (ng/mL)
6-hydroxywarfarin	110	80
7-hydroxywarfarin	50	45
8-hydroxywarfarin	40	20
4′-hydroxywarfarin	800	650
3′-hydroxywarfarin	255	125

	Alcohol Metabolites (ng/mL)
9R 11R - warfarin alcohol	40
9S 11S - warfarin alcohol	100
9R 11S - warfarin alcohol	1080
9S 11R - warfarin alcohol	350

stereoselective and similar to the one found in humans. Application of standardized scale-up techniques provided milligram quantities of specific metabolites. This technology was applied to producing the specific warfarin metabolites indicated in table 1-9.

Table 1-9. A summary of the novel metabolites obtained from the substrate warfarin using large scale production.

Researcher	Organism Used	Amount Biosynthesized
Wong *et al.* 1991	*Cunninghamella elegans* ATCC 36112	≈ 1.0 mg of 3′-Hydroxywarfarin
Rizzo *et al.* 1988	*Aspergillus niger* VI-X-172	95.0 mg of Warfarin α-diketone
Rizzo *et al.* 1989	*Cunninghamella bainieri* UI 3065	2.0 mg of 4′-Hydroxywarfarin
Griffiths *et al.* 1993	*Beauveria bassiana* IMI 12939	mg amounts of → 4′-Hydroxywarfarin → Warfarin α-diketone

1.3.4 Use of Cell Lines Expressing Human Cytochrome P_{450} cDNA.

The final *in vitro* technique considered is the use of cells containing and expressing human cytochrome P_{450} cDNA to study drug metabolism. With the advancements made in molecular biology it is now possible to utilize mammalian (Gonzalez *et al.* 1991, Dai *et al.* 1993), bacterial (Nishimoto *et al.* 1993, Gillam *et al.* 1993), and yeast (Renaud *et al.* 1993, Wiseman 1993) cells possessing minimal endogenous cytochrome P_{450} activity, expressing a specific cytochrome P_{450} cDNA. A bank of these cells can be screened to determine whether a drug is a substrate for a particular cytochrome P_{450}. By identifying and quantifying the products biosynthesized from each cell line a metabolic profile can be pieced together that may reflect the *in vivo* situation.

This technique was used to study warfarin metabolism and explore possible drug interactions *in vitro*. Rettie *et al.* (1992) used 11 cytochrome P_{450}

enzymes expressed in a human hepatoma derived cell line, called $HepG_2$ to identify the specific forms of human liver cytochrome P_{450} involved in warfarin toxicity. It appears that *in vivo* a high affinity S-warfarin-7-hydroxylase is responsible for terminating warfarin's biological activity. Inhibition of the formation of this metabolite in humans is known to be a cause of several of the drug interactions experienced clinically. After testing a range of human enzymes (table 1-10), only the cell line expressing CYP2C9 displayed the regioselectively and stereoselectively deemed responsible for metabolizing this drug *in vitro*.

An important mechanism responsible for the occurrence of clinically significant drug interactions is an inhibition of the drug's clearance by suppression of its biotransformation. Therefore, by identifying the specific cytochrome P_{450}, it becomes possible to screen for specific interactions. By incubating the cell line expressing the cytochrome P_{450} of interest with the drug of concern it is possible to determine whether a drug is a suitable substrate for an expressed enzyme. In addition, by setting up a data base containing this information, a search can be made for other possible interactions. This is much safer than finding an interaction after a drug has been released onto the market.

Table 1-10. The expressed human cDNA tested for an ability to metabolize the metabolic probe warfarin

Control $HepG_2$ Cells	CYP2D6 *
CYP1A2 **	CYP2E1 *
CYP2A6 *	CYP2F1 *
CYP2B7 *	CYP3A4 **
CYP2C8 *	CYP3A5 *
CYP2C9 ***	CYP4B1 *

* Same activity as control cells
** Elevated activity
*** Significant amount of activity

Therefore, with warfarin metabolism, a drug that has a high affinity for CYP2C9 might be considered a candidate for causing warfarin toxicity.

In conclusion, the data presented demonstrated that none of the preceding *in vitro* techniques provided a metabolic profile for warfarin identical to the one generated *in vivo*. In addition, on their own merit, none could reliably replace the use of animal models. Therefore, when testing the metabolism of a new therapeutic agent, the method(s) utilized will be determined by the information needed. Consequently, if an initial metabolic profile is required a microbial model is a rapid and inexpensive technique that could generate this information. To investigate this further, the objective of this thesis was to demonstrate how microorganisms can be used to achieve this goal. More specifically, the fungal metabolism of *rac*-mexiletine and *rac*-propranolol was examined in detail to demonstrate that microorganisms can provide useful information about human oxidative drug metabolism.

2.0 Materials and Methods

2.1 Chemicals, Reagents, and Equipment

Solvents:

BDH (Toronto, Canada)
  - HPLC grade chloroform.
  - Diethyl ether (anhydrous).

Baxter (Muskegon, MI, USA)
  - HPLC grade hexane.

Mallinckrodt (Paris, KY, USA)
  - HPLC grade water.
  - HPLC grade methanol.

Solvents were used without further purification.

Acids, Bases, and Salts

Fisher (Fair Lawn, NJ, USA)
  - Sodium chloride, Sodium bicarbonate, sodium hydroxide, potassium chloride, potassium phosphate (monobasic), potassium phosphate (dibasic), calcium chloride, and sodium phosphate.

BDH (Toronto, Canada)
  - Hydrochloric acid, L-ascorbic acid, maleic acid and acetic acid.

Commercial Buffers

Fisher (Fair Lawn, NJ, USA)
  - Tris.

Sigma (St. Louis, MO, USA)
  - HEPES.

Aldrich (Milwaukee, WI, USA)
  - MOPS.

Pharmaceuticals

Boehringer Ingelheim (Burlington, Canada)
  - R-(-), S-(+), and *rac*-Mexiletine, hydroxymethylmexiletine, and *p*-hydroxymexiletine.

Astra (U.K.)
  - S-(+) and *rac*-tocainide.

Ayerst (Montreal, Canada)
  - *rac*-Propranolol and *rac*-4-hydroxypropranolol.

Aldrich (Milwaukee, WI, USA)
  - R-(+), and S-(-)-propranolol, and *rac*-pindolol.

Synthesized in house.
  - Acetylated *rac*-mexiletine, acetylated *rac*-hydroxymethylmexiletine, acetylated *rac*-*p*-hydroxymexiletine, and acetylated *rac*-tocainide.
  - *rac*-Preprenalt and *rac*-prenalterol.
  - Prenalterol (isolated from fermentation media).
  - Deuterated S-(+), R-(-), and *rac*-propranolol.

Microbial Growth Media

Difco (Detriot, MI, USA)
  - Bacto-agar, casamino acids, czapec-dox broth, malt extract broth, Sabouraud dextrose broth, and yeast extract.

BDH (Toronto, Canada)
  - Peptone, D-glucose.

Baltimore Biologicals Ltd. [BBL] (Cockeysville, MD, USA)
  - Trypticase soy broth.

Mead Johnson (Mississauga, Canada)
  - Mixed cereal pablum.

Miscellaneous Chemicals

- S-(+)-1-(1-Naphthyl)ethyl isocyanate (NEIC) (Aldrich).
- Analytical grade triethylamine (Fisher).
- Butylamine (Aldrich).
- AquaSil [Octadecyltrialkoxysilane] (Pierce, Rockford, IL, USA).
- Glucose (Trinder) test kit (Sigma).
- Liquid nitrogen (Slowpoke, University of Alberta, Edmonton, Canada)
- Glycerol (BDH).
- Dimethyl sulfoxide (Mallinckrodt).
- Sodium dithionite (BDH).
- 4-Dimethylaminopyridine (Aldrich).
- Dithiothreitol (Sigma).
- D-Sorbitol (Sigma).
- Ethanolamine (Fisher).
- Triethylamine HPLC grade (Fisher).
- Extran 300 (Fisher).

Compressed Gases

- Nitrogen, Oxygen, and Carbon Monoxide (Union Carbide Canada Ltd., Edmonton, Canada).

Chromatography

Waters (Mississauga, Canada)
  - Model 590 pump.
  - Model 712 WISP.
  - Model 470 scanning fluorescence detector.
  - Model 486 tunable ultraviolet (U.V.) absorbance detector.
  - Model 600E system controller.
  - Model 717 autosampler.
  - Model 590 programmable solvent delivery module.
  - Model 745B integrator
  - Baseline 810 integration program.
  - Millenium integration program Ver. 2.0.

Technical Marketing Assoc. (Edmonton, Canada)
  - Applied Biosystems model 980 fluorescence detector.

NEC (Boxborough, MA, USA)
  - Powermate sx/16 computer.

Chromatography Columns

Phenomenex (Torrance, CA, USA)
  - Stainless steel Partisil 5 (250 × 4.6 mm I.D.).

Whatman (Clifton, NJ, USA)
  - Stainless steel Partisil 5 (250 × 4.6 mm I.D.).
  - Stainless steel Partisil 5 ODS-3 (125 × 4.6 mm I.D.).

Miscellaneous Equipment

- Milli-Q reagent water system (Millipore, Bedford, MA, USA).
- Disposable sterile 0.22 μmAcrodisc filters (Gelman Sciences, Rexdale, Canada).
- Amsco steam autoclave (Amsco, PA, USA).
- Dynac benchtop centrifuge (Becton, Dickinson and Co., Parsippany NJ, USA).
- Vortex mixer (IKA-Werk, Terochem, Edmonton, Canada).
- Orion 520A pH meter (Boston, MA, USA).
- Airlift bioreactor (300 mL) and 12 test tube nitrogen manifold (made by Technical Services, University of Alberta, Edmonton, Canada).
- Model L5-50 ultracentrifuge (Beckman, Mississauga, Canada).

- Type 40 fixed angle rotor (Beckman, Mississauga, Canada).
- 10 mL ultracentifuge tubes(Nalgene, Fisher, Fair Lawn, NJ, USA).
- Damon/IEC B20A centrifuge (c/o Fisher, Fair Lawn, NJ, USA).
- UV/Visible spectrophotometer series P48700 (Phillips).
- Controlled environment incubator-shaker, model G-25 (NewBrunswick Scientific Co. Inc., Edison, NJ, USA).
- Bioguard Model B60-112 Class II Cabinet (Baker Co., Sanford, ME, USA).
- Incubator (Blue M Electric Company, IL, USA).
- Model 601 and 602 gas flow meters (Matheson Gas Products, Edmonton, Canada).
- Potter-Elvehjem glass tissue homogenizer (Fisher, Fair Lawn, NJ, USA).
- Sterile, disposible, 50 mL centrifuge tubes (Fisher, Fair Lawn, NJ, USA).
- Drummond pipet aid (Fisher, Fair Lawn, NJ, USA).
- Branson 3200 sonnicator (Fisher, Fair Lawn, NJ, USA).
- Hemocytometer (Spencer Bright-Line, Fisher, Fair Lawn, NJ, USA).
- Oxford (200 ul) large orifice tips (Sherwood Medical, St Louis, MI, USA).
- Microtubes (1.5 mL) with screwcap closures (Sarstedt, St. Laurent, Canada).
- Orthomat microscope camera (Leitz and Wetzlar, Toronto, Canada).
- Oxygen meter (NewBrunswick Scientific Co. Inc., Edison, NJ, USA).
- Spectronic 20 spectrophotometer (Bausch and Lomb).
- VG Trio 2000 LC/MS (Fisons Instruments, Mississauga, Canada).
- Corning brand 150 mL milk dilution bottles (Fisher, Fair Lawn, NJ, USA).
- Micro Fernbach (25 mL) flasks (Fisher, Fair Lawn, NJ, USA).

2.2 Microorganisms Evaluation and Their Storage

Choosing a microorganism, and a method to store it, are the most important decisions to make prior to conducting a microbial biotransformation experiment. Perlman and Kikuchi (1977) have listed some general attributes an organism should possess in order to be considered a good candidate for experimentation (Table 2-1).

Based on literature precedents, there are a number of fungal organsims that meet these requirements. In addition to being genetically stable, they are also capable of performing biotransformation reactions that parallel those found in mammals. The fungi used for metabolic experiments are listed in table 2-2.

Table 2- 1. Attributes that should be considered when selecting a microorganism for a biotransformation experiment.

---

1. The strain must be genetically stable.
2. The strain must be amenable to long-term storage.
3. The strain must readily produce vegetative cells or spores.
4. The strain must grow vigorously and rapidly in the chosen media.
5. The strain must be free of contamination.

All cultures were obtained as maintenance slants from the University of Alberta Microfungus Collection and Herbarium (UAMH). Cultures were stored in the lab on Mixed Cereal Pablum agar maintenance slants kept refrigerated at 4°C. In order to maintain fungal viability, spores must be transferred to fresh media every six months. The following procedure was utilized to prepare all new maintenance slants:

Table 2- 2. A list of the fungi tested and used for the biotransformation experiments.

Organism	UAMH Identification	Additional Identification
Aspergillus niger	1240	ATCC 9142 [1]
Aspergillus flavus	2679	---------------
Cunninghamella echinulata	4144	ATCC 36190
Cunninghamella echinulata	4145	ATCC 9244
Cunninghamella echinulata	7368	ATCC 9246
Cunninghamella echinulata	7369	ATCC 26269
Cunninghamella echinulata	7370	ATCC 36112
Rhizopus stolonifer (syn. *nigricans*)	1947	---------------
Rhizopus oryzae	2665	PRL 2265 [2]

1 American Type Culture Collection
2 Prairie Regional Laboratories

I Equipment and Solutions Required

1. Large capacity Erlenmeyer flask (1.0 L) to prepare and autoclave the Mixed Cereal Pablum agar. Cereal grains are known to contain fungal spores. These have the potential to contaminate a storage culture. Therefore, when preparing maintenance agar, the mixed cereal agar solution's final volume should be no greater than 20% of the total flask volume. This creates a large surface to volume ratio which augments sterilization (30 min).

   Maintenance agar contains:

   - 10% Mixed Cereal Pablum (0.1 g/mL).
   - 3% Bacto agar (0.03 g/mL).

2. Sterile 10 mL culture tubes with appropriate caps.
3. Sterile 1, 5, and 25 mL pipettes and pipette aid.
4. Sterile 500 mL flask containing 250 mL of sterile hot (≈ 80°C) water.
5. Sterile trypticase soy broth (20 mL prepared according to label instructions).
6. Nichrome inoculating loop.

II Once the appropriate equipment and solutions have been sterilized, the maintenance slants were prepared in two steps.

Step 1] Preparing Slants (all work was done using aseptic technique in a laminar flow biohood).

1. Allow equipment and media to cool sufficiently to be handled without injury.
2. Using a sterile 25 mL glass pipette, cycle 20 mL aliquots of sterile hot water through the pipette to heat the glass. This prevents mixed cereal agar from solidifying inside the pipette.
3. With the heated glass pipette, remove 25 mL of sterile mixed cereal agar and dispense 4 mL into a 10 mL culture tube. A total of 6 culture tubes can be inoculated per withdrawal.
4. Once filled, cap the tube and lay it horizontally. After the agar solidifies, a slant is formed the length of the tube providing a large surface area for fungal growth.

Step 2] Preparing a Spore Suspension.

1. Pipette 5 mL of sterile trypticase soy broth onto the maintenance slant surface.
2. Using a sterilized nichrome inoculating loop, agitate the slant surface. This knocks the hydrophobic spores into the liquid medium facilitating their transfer to a new slant.
3. Remove a 1.0 mL aliquot of the spore suspension from the slant, and inoculate a new tube with 0.2 mL (all new storage cultures must be properly labeled).
4. Incubate the inoculated slants for 72 hours at 27° C. During this time the spores will germinate producing new mycelia and spores.

A maintenance slant will remain viable for approximately six months when stored at 4°C. At that time spores should be transferred to fresh media in order to prevent a loss in viability. These slants were the starting point for all biotransformation experiments performed.

2.3 Initiating Submerged Fungal Growth

Fungi exhibit unique morphologies when grown in different environments. For example, when *C. echinulata* was inoculated onto the surface of solidified media, it grew out over the entire surface. Under these conditions it would produce asexual spores inside monosporic sporangiola (Hawker *et al.* 1970) to insure survival. When *C. echinulata* was grown in submerged liquid culture, its morphology and growth characteristics changed. Fungi, which are hydrophobic by nature, can grow as diffuse mycelia in liquid culture. However, *C. echinulata* grew in the form of distinct pellets whose morphology was altered by changing pH. This diversity made it a difficult fungus to deal with when attempting to generate reproducible results in a laboratory setting. Therefore, when conducting a biotransformation experiment a standardized procedure simplifying the transition from surface culture to submerged liquid culture in a consistent manner was desirable. To achieve this, the following general two phase procedure was utilized when fungal organisms were evaluated for their ability to biotransform a xenobiotic. Phase I growth was intended to generate a large biomass of mycelia. Once this had been achieved (36 hours) the spent medium was removed, the mycelia washed with phosphate buffer (pH 7.0) and resuspended in fresh growth medium containing the xenobiotic under study. This was known as phase II. The cultures were allowd to incubate for the length of time required to metabolize the test compound (monitored by HPLC). The following is a summary of the standard protocol utilized to conduct a biotransformation experiment. This procedure, which proved to be reproducible was refined and developed over time.

2.3.1 Submerged Culture Technique

Originally, phase I cultures were initiated by adding 2.0 mL of sterile distilled water to a maintenance slant which was sealed and shaken vigorously. The resulting spore suspension was recovered and 1.0 mL was transferred to a metal-capped 125 mL Erlenmeyer flask with 25 mL of medium. After using this method, it became apparent that achieving consistent fungal growth from experiment to experiment would be difficult. It appeared the inconsistency was caused by a difference in the number of spores contained within individual maintenance slants. A solution to this problem was to utilize a hemocytometer and determine the exact number of spores contained within a maintenance slant to standardize the inoculum. This approach gave a physical account of the number of spores but offered no information concerning viability. However, preparing a "standardized" inoculum gave consistent results for all biotransformation experiments performed. Generally, when an inoculum of 48,000 spores/mL was used, 8.0 grams (wet weight after centrifugation) of mycelia could be produced in 36 hours.

2.3.1 Making a Fungal Spore Suspension

I Equipment and solutions required for generating a spore suspension.

1. Trypticase soy media (25 mL prepared according to label instructions).
2. Sterile 25 mL micro Fernbach flask.
3. Sterile .1 mL, 1.0 mL, and 10 mL pipettes and pipette aid.
4. Sterile 10 - 200 µL Oxford large orifice pipette tips.
5. 5.0 - 50.0 µl hand pipetter.
6. Nichrome inoculating loop.
7. Hemocytometer.
8. Sonicator.
9. Fungal maintenance slant.
10. Microscope.

II Once the appropriate solutions and equipment had been sterilized, the spore suspension was made using the following procedure (all work was performed using aseptic technique in a laminar flow biohood).

1. Aseptically transfer 6.0 mL of trypticase soy broth to a maintenance slant containing the organism being cultured.
2. Using a nichrome inoculating loop, gently agitate the slant surface (Davis *et al.*, 1988) to dislodge the hydrophobic spores and force them into the aqueous medium. If the spores are hydrophobic (e.g. *Aspergillus niger*), surfactant (e.g. Tween 80) can be added to the trypticase soy broth (2% w/v) thereby increasing dispersibility.
3. Remove the trypticase soy broth from the slant and transfer it to a sterile micro Fernbach flask (50 mL).
4. Sonicate micro Fernbach flask for 5.0 min to generate an even suspension.
5. Aseptically remove, using Oxford pipette tips, 100 µL of the spore suspension and transfer it to 900 µL of water in a 1.5 mL microfuge tube.
6. The diluted spore suspension is vigorously vortexed. Transfer 10 µL of this suspension to the hemocytometer.

7. Using the microscope 10x magnification lens, count the total number of spores in 5 squares (squares are designated with an X) and add them together.

Hemocytometer Grid

X		X
	X	
X		X

8. To convert this count into the number of spores per mL, in the micro Fernbach flask, use the following equation:

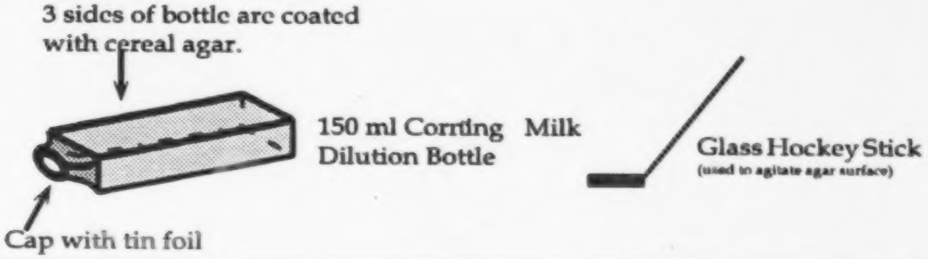
$$\textbf{Spores per mL} = \frac{\text{Total \# of spores counted} \times 10 \times \text{Dilution factor}^{*}}{5}$$

* Dilution factor is 1:10,000 when working with *C. echinulata*. The exact dilution must be determined for each strain of fungus utilized.

To simplify this procedure when working with *C. echinulata,* multiply the total number of spores counted by 20,000. The total will represent the number of spores per mL in the micro Fernbach flask.

The resulting suspension served as the source of spores utilized to initiate phase I of a biotransformation experiment. A problem encountered when large numbers of spores were required was that up to eight maintenance slants had to be used to prepare a stock spore suspension. This proved to be very time consuming. A solution to this problem was to prepare larger maintenance slants which we called spore seed maintenance flasks. These were prepared in 150 mL Corning Brand milk dilution bottles. Three sides of a bottle were lined with mixed cereal agar by adding three successive 25 mL aliquots of liquefied medium. This created a large surface area for the fungi to grow and resulted in

Figure 2- 1. Milk dilution bottle (150 mL) used to prepare a spore seed maintenance slant. A glass "hockey stick" was used to dislodge the spores.


3 sides of bottle are coated with cereal agar.
150 ml Corrting Milk Dilution Bottle
Glass Hockey Stick
(used to agitate agar surface)
Cap with tin foil

a large number of spores being generated in a small space. A 12 inch glass "hockey stick" (sterilized with ethanol) was used to agitate the agar surface in order to recover the spores.

2.3.1.1 Long-Term Preservation

A successful biotransformation experiment depends on the availability of a reliable and reproducible seed inoculum. Maintenance slants were not the only means by which fungal spores were preserved. Freezing a spore suspension at minus 80°C was another technique employed. At low temperatures spores can be protected from the cold by adding the cryoprotective agent glycerol. Autoclaved glycerol (10% v/v) was added to the spore suspensions to guard against deterioration. The glycerol spore suspension mixture was stored as 1.0 mL aliquots in sterile micro tubes with screw cap closures. These were labeled and stored at minus 80°C. Using C. *echinulata* 4145 as a test organism, spores remained viable for two years.

2.3.2 Phase I Fungal Growth

An important consideration when starting a biotransformation experiment was the types of media to use. Microorganisms have specific nutritional requirements that must be met to achieve growth. To accommodate these varied demands, numerous commercial formulations are available. By means of literature precedent and trial and error, it was found the following two (Table 2-3) provided for rapid fungal growth, a large biomass and mycelia capable of biotransformation. Trypticase soy broth was used as indicated on the label instructions. Yeast extract broth composition was determined by comparing media formulations used by other laboratories working with C. *echinulata*. The intent was to utilize the minimum number of components necessary for rapid growth while providing a biomass capable of biotransformation. Other media used are listed in table 2-4.

Table 2- 3. The majority of biotransformation experiments were conducted using one of these two medium preparations.

A. **Trypticase soy broth** pH 7.3	
- Trypticase soy powder	30.0 g/L
B. **Yeast extract broth** pH 7.2	
- Yeast extract	8.0 g/L
- NaCl	4.6 g/L
- Sucrose	20.0 g/L
- Monopotassium phosphate	3.75 g/L
- Disodium phosphate	7.09 g/L

After a growth medium was chosen, phase I of the biotransformation experiments were conducted in 125 mL Erlenmeyer flasks containing 25 mL of media (flasks were capped with metal culture tube closures). To decrease the risk of contamination, the medium was prepared in bulk followed by transfer of 25 mL to individual 125 mL flasks (small volumes in a large surface area). These were autoclaved at 122°C (22 psi) for 17 min in an Amsco general purpose steam autoclave. After sterilization and cooling to room temperature, aliquots of the spore suspension (generally 1.2 million spores per 25 mL of media) were added

Table 2- 4. Additional medium formulations utilized for biotransformation experiments.

A. Czapek dox broth pH7.3	
- czapek dox powder	35.0 g/L
B. Sabouraud dextrose broth pH 5.7	
- sabouraud dextrose powder	30.0 g/L
C. Malt extract broth pH 4.7	
- malt extract powder	20.0 g/L
D. Casamino acid broth pH 7.2	
- casamino acids	8.0 g/L
- NaCl	4.6 g/L
- Sucrose	20.0 g/L
- Monopotassium phosphate	3.75 g/L
- Disodium phosphate	7.09 g/L
E. Peptone broth pH 7.2	
- peptone	8.0 g/L
- NaCl	4.6 g/L
- Sucrose	20.0 g/L
- Monopotassium phosphate	3.75 g/L
- Disodium phosphate	7.09 g/L

aseptically to each flask. These were incubated at 27°C in a New Brunswick Scientific Co. Model G-25 gyratory shaker equipped with 45° angle brackets (created turbulent flow) and operated at 250 rpm. Phase I cultures were incubated for a minimum of 36 hours. All incubation flasks were coated with AquaSil siliconizing fluid to maintain submerged culture homogeneity. Fungi used for these biotransformation experiments would attach to the glass surface at the liquid/air interface. Coating the interior surface of an Erlenmeyer flask prevented this and encouraged homogeneous growth. Erlenmeyer flasks were coated after every 10 autoclavings.

Preparation of the medium was standardized using the same electronic balance, pH meter, and glassware. Double distilled water from a Millipore Milli-Q reagent water system equipped with a carbon filter, two ion exchange cartridges, an organ-x filter, and a 0.22 µm filter, was used routinely for all experiments. This provided water whose purity was measured at 16 ohms. All glassware was washed with Extran 300, rinsed with tap water, and finally rinsed with Milli-Q water.

2.3.2.1 Wet Weight Determination

In our laboratory, wet weight was defined and standardized using the following procedure:

1. Weigh sterile 50 mL disposable centrifuge tubes.
2. Harvest fungus from 125 mL Erlenmeyer flask by transferring contents into weighed 50 mL sterile centrifuge tubes.
3. Centrifuge at 900 x g for 5 min.
4. Decant supernatant leaving a loosely packed pellet of cells.
5. Weigh.
6. Subtract tube weight from final weight.
7. The mass obtained was defined as wet weight.

2.3.2.2 Coating Glassware With AquaSil (Octadecyltrialkoxysilane)

To prevent the fungus from attaching its hyphae to the interior glass surface, each flask was coated with a siliconizing agent. The commercial preparation of choice was AquaSil because it was water soluble and could be applied using the following procedure:

1. A 0.2% solution of AquaSil was prepared according to the label instructions (one part AquaSil fluid plus 99 parts water (w/w) yields a 0.2% solution).
2. Enough solution was prepared to fill the flasks being treated (100 mL of solution per 125 mL flask) for a 24 hour reaction period.
3. Dispose of the used solution and let the flasks air dry for 24 hours.
4. This procedure prevented fungi from attaching to the glass surface for a minimum of 10 autoclavings. When a ring of growth started to appear, the flasks were re-coated.

2.3.3 Phase II Fungal Growth

As was stated previously, phase I growth was intended to rapidly generate a large biomass of mycelia. The purpose of phase II growth was to utilize the biomass for biotransformation experiments. Initially, phase II cultures were prepared by aseptically homogenizing phase I cultures (Waring blender with mini sample cup container) into a fine mycelial suspension, portions of which were added to fresh medium (5% inoculum). However, at best, this method generated limited phase II growth. Therefore, another procedure was implemented based on methods used with bacteria. After 36 hours incubation the contents of a phase I 125 mL Erlenmeyer flask were transferred to a disposable, sterile, 50 mL centrifuge tube. This was centrifuged at 900 x g for 5 min. The spent medium was poured off and 25 mL of sterile phosphate buffer (pH 7.0) was added to wash the mycelial pellet. After centrifugation (5 min at 900 x g) the buffer was decanted and the mycelial pellet resuspended in 25 mL of fresh sterile medium (vortex for 30 s). The suspension was aseptically transferred back to the 125 mL Erlenmeyer flask. The advantage of this procedure was that it utilized all the biomass created in phase I (generally between 6.0 and 8.0 g wet weight). The drug substrate was added at this point by dissolving it in water and dispensing suitable aliquots through a disposable

0.22 µm Acrodisc filter to a final concentration of between 50 and 350 µg/mL. The incubations were allowed to continue for up to seven days.

All experiments included a culture control which was prepared by incubating the microorganism in the absence of substrate. Samples of the culture were also periodically plated on mixed cereal agar plates to check for contamination. In order to determine stability of the test compounds under incubation conditions substrate controls were prepared by incubating the drug in sterile medium for the duration of the experiment.

2.3.4 Sampling Procedures

Biotransformation experiments were performed using the test compounds *rac*-1-isopropylamino-3-phenoxy-propan-2-ol (preprenalterol), *rac*-mexiletine, and *rac*-propranolol. Metabolic pathways used to eliminate these drugs in humans are well established and this information was used to our advantage. By obtaining authentic metabolic standards from appropriate drug companies, we were able to develop HPLC methods for their analysis. Typically, it was possible to measure 50 ng/mL of drug and metabolite in a fermentation broth.

If numerous samples were required from a culture flask the volume of growth media will eventually be depleted. An approach used to circumvent this problem was to remove small volumes of fermentation broth (150 µL) at each sampling time. As a result, a large number of samples could be removed without adversely affecting the overall volume of liquid inside the flask. In addition, the sensitivity of our analytical methods allowed the use of even smaller aliquots (25 µL) of a timed sample for analysis.

The materials used to sample a biotransformation experiment included a 20 - 200 µl pipette, sterile Oxford pipette tips (200 µL), labeled 1.5 mL microfuge tubes, and ethanol. The procedure involves removing the 125 mL Erlenmeyer flasks from the gyratory shaker and placing them in the biohood. The digital pipette was wiped down with ethanol and a sterile pipette tip was attached (tips are kept sterile in an autoclavable holder). Using aseptic technique, 150 µL of broth were removed from the Erlenmeyer flask and transferred to a microfuge tube. Large orifice pipette tips were used because their design was amenable to working with filamentous fungi. Once the sample tube had been capped and centrifuged for 5 min, it was stored -20 °C until analyzed. This protocol allowed a large number of samples to be withdrawn over time (biotransformation experiments could last for one week). The information generated could be used to construct a concentration versus time metabolic profile indicating the time of maximum metabolite production. This was important to our goal of biosynthesizing semi-preparative amounts of metabolites.

2. 4 Analytical Procedures

2.4.1 Glucose Analysis

When using a microorganism as a tool to perform specific tasks, it can be viewed as a black box, taking in food and generating products. Moreover, an advantage to the use of microorganisms is that the more information that is gained concerning their growth, the easier it is to optimize product formation. One aspect of fungal development which was of interest to us was the rate of growth in submerged culture. In an unrestricted system under optimal

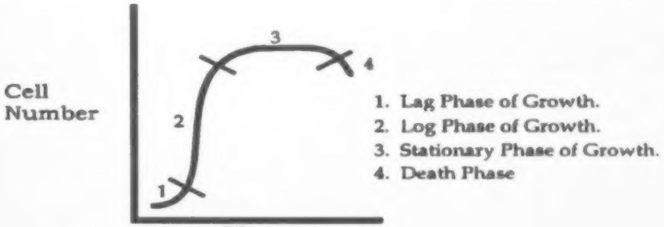


Figure 2- 2. Diagrammatic representation of the four phases of microbial growth in an unrestricted system under optimal conditions.

conditions, an organism's growth should follow a certain pattern (Figure 2-2). During the four phases of growth it is likely that an organism will express different enzymes. The enzyme(s) of interest to us was cytochrome P_{450}. We wished to determine whether it was expressed during a specific time or phase of growth. Traditionally, growth rates were determined using dry weight as an indicator. However, it was difficult to generate this information with *C. echinulata*. Another method we considered was to determine glucose consumption and correlate this with metabolite production.

To determine glucose concentrations we used Sigma's Glucose Trinder reagent kit. This was an indirect test measuring quinoneimine dye production at 505 nm. The procedure involved making a stock glucose solution (20 g/L) in the growth medium to be tested. This was diluted to yield four test solutions having final concentrations of 1.0 mg/mL, 3.0 mg/mL, 5.0 mg/mL, and 7.0 mg/mL.

Standard Concentrations	Absorbance
(mg/mL)	505 nm
0.0	0.0
1.0	0.13
3.0	0.27
5.0	0.44
7.0	0.58
$r^2 = 0.9966$	

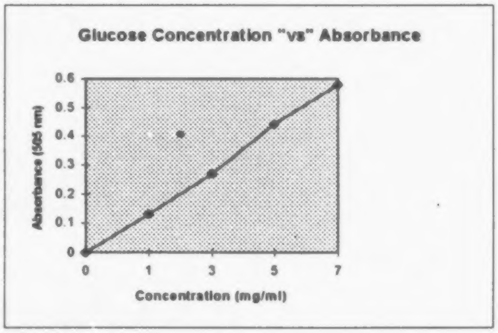


Figure 2- 3. A typical line generated from standard solutions prepared in yeast extract broth.

These were further diluted 1:10, along with the unknown microbial broth samples. For each sample tested, 10 µL was added to a separate 2.5 mL Trinder enzyme solution. These were incubated at 37°C, for 10 min and the absorbance was measured using a Spectronic 20 set at 505 nm (Figure 2-3).

2.4.2 Oxygen Analysis

Microorganisms have the ability to express numerous proteins in response to environmental stimuli through transcriptional and translational regulation. Therefore, an organism can respond directly to its immediate surroundings by expressing the enzymes necessary for growth. It is possible to manipulate these control mechanisms and induce an enzyme system of interest. Poyton *et al.* (1992) and Ishidate *et al.* (1969) demonstrated that oxygen concentration regulated cytochrome c and cytochrome c oxidase genes in yeast. We wanted to determine if decreased oxygen levels could increase the concentration of cytochrome P_{450} in *C. echinulata* 4145.

The oxygen concentration in the liquid media was determined indirectly using a New Brunswick Scientific dissolved oxygen meter, oxygen electrode, G-25 gyratory shaker, purified nitrogen plus oxygen gases, modified steel cap closures (Figure 2-4), and tygon tubing. The assumption made when designing this experiment was that if gas flow into all 125 mL Erlenmeyer flasks was identical the dissolved oxygen concentration in every flask will be the same (Figure 2-5). Therefore, flasks used were set up in series and one was designated the oxygen control (Figure 2-6). The New Brunswick Scientific G-25 gyratory shaker was

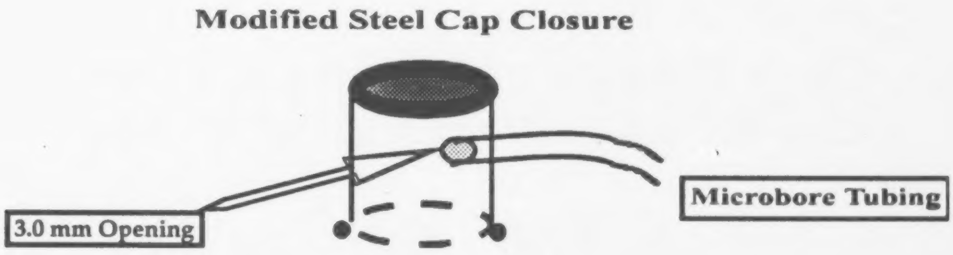


Figure 2- 4. Modified steel cap closure with 1 cm opening into which surgical microbore tubing is inserted.

equipped with a gas line and tapered fitting that connected the shaker's interior

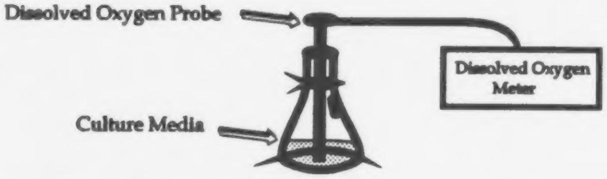

Dissolved Oxygen Probe
Dissolved Oxygen Meter
Culture Media

Figure 2- 5. Indirect oxygen concentration measurement using one test flask from a group that has been set up in series.

with the outside environment. Using a Y connector and two independent Matheson flow meters, the nitrogen plus oxygen gas tanks were connected to the gyratory shaker's exterior gas line. A 0.5 inch tygon tube with one end plugged was attached to the tapered fitting inside the shaker. 23 Guage needles with 22 micron filters

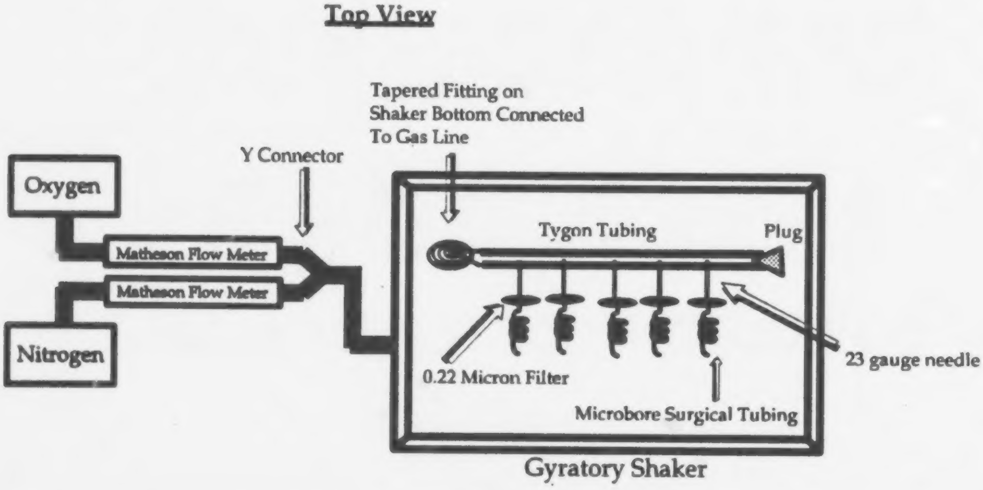

Top View
Tapered Fitting on Shaker Bottom Connected To Gas Line
Y Connector
Oxygen
Matheson Flow Meter
Matheson Flow Meter
Nitrogen
Tygon Tubing
Plug
0.22 Micron Filter
23 gauge needle
Microbore Surgical Tubing
Gyratory Shaker

Figure 2- 6. Top view of how the 125 mL Erlenmeyer flasks are set up in series. They are attached to the microbore surgical tubing.

attached were inserted into the tygon tube. Autoclavable, surgical microbore tubing was attached to one end of the filter unit and the other end was inserted through the opening in the steel cap closure into the 125 mL Erlenmeyer flask. This allowed control of the gas composition and flow into all flasks. The dissolved oxygen concentration, which was a direct consequence of the gas flow, was controlled by the flow meters.

2.4.3 Cytochrome P_{450} Determination

To examine fungal growth and enzyme production, an experiment was designed to isolate and quantify *C. echinulata's* cytochrome P_{450} enzyme system. Our objective was to quantify and optimize cytochrome P_{450} levels in order to maximize (mammalian) metabolite production.

2.4.3.1 Disrupting The Fungal Cell Wall

A cell wall is structurally important to a fungus because it determines the organism's characteristic shape. If the wall is removed the resulting protoplasts are spherical in nature. Even though the gross chemical composition of fungal cell walls differs between taxonomic groups, there are some common features. Fungal cell walls contain a mixture of fibrous and amorphous components. The fiber is usually made from chitin which is a β-1,4-N-acetylglucosamine. This forms a network of microfibrils possessing great tensile strength. The amorphous portion includes proteins, mannans, and glucan polymers. Overall, these components create a structure which is extremely resistant to mechanical disruption yet compliant enough to allow the organism to interact with its environment (Kuhn and Trinci 1990).

We found the most difficult task involved in isolating fungal cytochrome P_{450} was breaking open the fungal cell in a simple and reproducible manner. The following is a list of the various methods attempted:

1. Homogenize the mycelia in a Warring blender.
2. Sonicate the mycelia, at full power, for 1 min bursts (8x) with a Heat Systems model XL2010 sonnicator. This technique was also repeated with the inclusion of 0.1 mm glass beads.
3. Homogenize with a hand held Potter-Elvehjem style glass tissue grinder.
4. Homogenize with a VirTis, VirTishear mechanical homogenizer (complements of Canberra Packard, Canada)
5. Generate protoplasts from 22 hour mycelia using the following enzyme preparations:

    Lyticase (Sigma)
        - Used 500 units in 5.0 mL.
    Trichoderma harzianum enzyme suspension (Sigma)
        Used 0.040 g in 2.0 mL.
    Helix pomatia enzyme suspension (Sigma)
        - Used 260 μL per flask.

6. Lyophilize 36 hour mycelia and grind it with sea sand.
7. Use liquid nitrogen (complements of Slowpoke, University of Alberta) to freeze mycelia (contained 0.1 mm glass beads) in a ceramic mortar. This was ground by hand with a ceramic pestle.

Of these techniques, the liquid nitrogen and 0.1 mm glass beads worked best. Unfortunately, this was not always the most convenient way to disrupt fungal cell walls because liquid nitrogen supplies were uncertain. Therefore, an alternative was to use the Potter-Elvehjem glass tissue grinder which provided comparable results.

2.4.3.2 Buffering Systems

The original method used for isolating cytochrome P_{450}, from mammalian liver preparations, was developed by Tsuneo Omura and Ray Sato (Omura and Sato 1964). Since that time, numerous modifications have been made to their technique. Changes were introduced to deal with cytochrome P_{450} diversity and to accommodate the cell, tissue, organ, or organism from which it was isolated.

It is important to re-emphasize that cytochrome P_{450} is an enzyme complex consisting of three parts. For the enzyme to be active after isolation, extreme caution must be taken to ensure the protein is not denatured and the lipid component remains intact. To achieve this goal, many buffering recipes were developed to stabilize the cytochrome P_{450} enzyme complex. Some typical examples are listed in table 2-5.

Table 2- 5. Buffering recipes developed to isolate cytochrome P_{450} from different microorganisms and mammalian liver.

Fusarium oxysporum (Shouu *et al.* 1989)	***Saccharomyces cerevisiae*** (Wright *et al.* 1989)
- 0.2mM PMSF [1] - 0.75 M Sorbitol - 10% Glycerol - 50 mM KH_2PO_4 buffer (pH 7.2)	- 1 mM DTT [2] - 1mM EDTA [3] - 200 μM PMSF [1] - 1% Sodium cholate - 20% Glycerol - 100mM KH_2PO_4 buffer (pH 7.2)
Aspergillus flavus (Hamid 1987)	***Rhizopus nigricans*** (Breskvar 1983)
- 0.25 M Sucrose - 0.01 M EDTA [3] - 0.01 M Glutathione - 0.5% Sodium deoxycholate - 0.067 M KH_2PO_4 buffer (pH 76.9)	- 0.1 M KCl - 30% Glycerol - 1 mM DTT [2] - 0.5% Sodium deoxycholate - 0.5 M sucrose - KH_2PO_4 buffer (pH 5.5)
Cunninghamella echinulata (Cerniglia *et al.* 1978)	**Mammalian Liver** (Coon *et al.* 1982)
- 1 mM DTT [2] - 1mM EDTA [3] - 0.25 M sucrose - 0.2% Triton DN 65 - 100mM KH_2PO_4 buffer (pH 7.5)	To a final volume of 100 mL add: - 44.5 mL Glycerol - 14.8 mL 50 mM Potassim citrate buffer (pH 7.6) - 14.8 mL 1 M KCl - 1.5 mL 0.1 M DTT [2] - 7.6 mL 10% Sodiun deoxycholate - 16.8 mL 0.25 M sucrose

1. Phenylmethylsulfonyl fluoride
2. Dithiothreitol
3. Ethylenediamine tetraacetic acid

In general, two buffer solutions were used to isolate cytochrome P_{450} from *C. echinulata* 4145. The first solution, called "cracking buffer" (Table 2-6), was utilized to break open the fungus. It was designed to furnish an environment similar to the one that existed in the fungal cytoplasm. In addition, it contained ingredients whose function was to minimize protein denaturation. After enzyme isolation *via* centrifugation, a resuspension buffer was used to re-solubilize cytochrome P_{450}. The enzyme preparation was now ready for activity testing or spectral analysis.

Table 2- 6. Contents of the cracking and resuspension buffer used to isolate cytochrome P_{450} from *C. echinulata* 4145.

Cracking Buffer
- 0.1 M MOPS[3] pH 7.2[1] - 0.25 M Sucrose[2] - 1.0 mM DTT - 1.0 mM EDTA - 20% Glycerol
Resuspension Buffer
- 0.1 M MOPS[3] pH 7.2 - 0.25 M Sucrose - 1.0 mM DTT

1. Can substitute with 50 mM Tris pH 7.2
2. Can substitute with 0.65 M Sorbitol
3. 3-(N-morpholino) propanesulfonic acid

2.4.3.3 Isolating Fungal Cytochrome P_{450}

Isolating fungal cytochrome P_{450} was initiated by preparing phase I growth using the methods described previously. Once there was sufficient biomass (determined for each experiment), the contents of a 125 mL Erlenmeyer flask were poured into a 50 mL centrifuge tube. This was centrifuged at 900 x g for 10 min at which time the supernatant was poured off. The mycelium was washed by resuspending it in 25 mL of 0.1 M MOPS (pH 7.2) and vortex mixing for 1 min.

After centrifuging at 900 x g for 10 min, the supernatant was decanted, and the fungal pellet weighed.

The following work was done in a cold room (2°C) with chilled cracking and resuspension buffers. Low temperatures inhibit fungal proteases, released during cell disruption, which can digest the enzyme of interest. Washed mycelia were resuspended in 10 mL of cracking buffer and the contents poured into a Potter-Elvehjem tissue grinder tube (glass). Using a glass pestle, the mycelia was ground until all clumps were pulverized into a suspension. The homogenate was poured into a 50 mL centrifuge tube and spun at 6400 x g for 20 min. This removed cell wall components, nuclei , and mitochondria. The supernatant was transferred to a 10 mL ultracentrifuge tube and spun at 40,000 rpm (105,000 x g) for 60 min. If access to an ultracentrifuge was not possible, the microsomal pellet could be isolated using $CaCl_2$ precipitation (Kappeli *et al.* 1982). The supernatant was poured off and the pellet reconstituted with 1.0 mL of resuspension buffer. Both methods generate a small, oily, brown sediment known as the microsomal pellet. This can be used for spectral analysis or activity testing. Normally, the microsomal pellet had to be scraped out of the centrifuge tube and placed in a 1.0 mL hand held tissue grinder where it was ground into a suspension.

2.4.3.3.1 Spectral Analysis

Cytochrome P_{450} derives its name from the ability to absorb light at 450 nm after being exposed to carbon monoxide. An attempt was made to utilize this characteristic along with the following equation to quantitate the amount of enzyme present in fungal mycelium.

$$A = \varepsilon c t$$

A = absorbance at 450 nm
c = concentration (mol/l)
t = thickness of light path (1.0 cm)
ε = molar extinction coefficient
P_{450} = 91 liter $mmol^{-1}$ cm^{-1}
(Omura and Sato 1964)

A cytochrome P_{450} spectrum was generated by pouring the reconstituted microsomal pellet into a cuvette and bubbling nitrogen gas through it. A spatula tip of sodium dithionite (reduces P_{450}) was added and mixed to generate baseline absorbance with the spectrophotometer. Carbon monoxide gas was then added by bubbling it through the reduced microsomal pellet for three minutes. If intact cytochrome P_{450} was present a peak should appear at 450 nm when the absorbance of this preparation was measured (Figure 2-7).

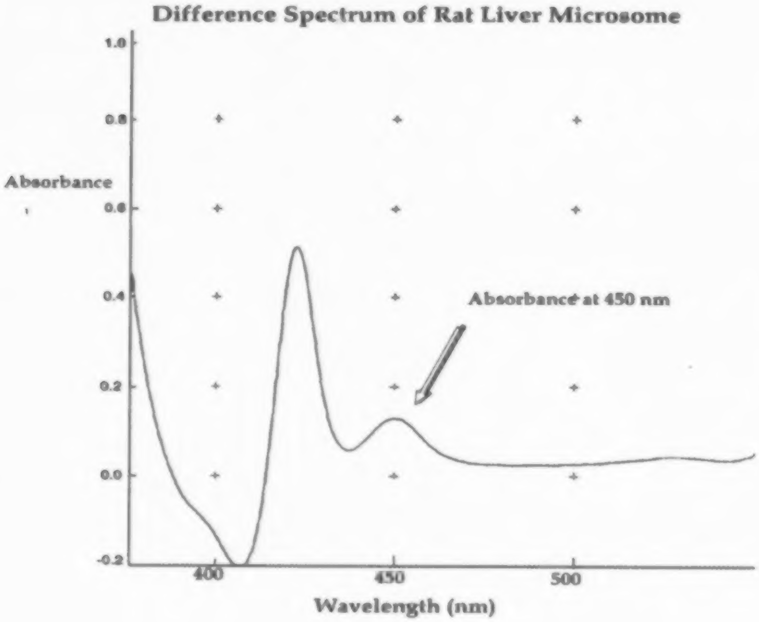


Figure 2- 7. Difference spectrum generated from a rat liver (5.0 g) prepared using Cerniglia's method (Cerniglia *et al.* 1978).

2.4.3.3.2 Activity Testing

The microsomal pellet was tested for activity by determining if it was capable of metabolizing *rac*-mexiletine *in vitro*. The procedure used was developed by Su *et al.* (1993). Additions to this protocol included the use of 1.1

mL of fungal microsome (instead of human) and 0.7 mL of the drug *rac*-mexiletine (4.8 μg/mL) instead of imipramine.

2.5 High Performance Liquid Chromatographic Analysis (HPLC)

2.5.1 Stereospecific Analysis of *rac*-1-Isopropylamino-3-phenoxy-propan-2-ol (Preprenalterol) and *rac*-Prenalterol.

2.5.1.1 Chromatography

The HPLC system (Waters, Ontario, Canada) consisted of a model 590 pump, 712 WISP autosampler, and a model 745B integrator. Fluorescence detection (Applied Biosystems Model 980, Edmonton, Canada) was set at 220 nm for excitation and 350 nm for emission. The mobile phase consisted of hexane-chloroform-methanol (76:22:2 v/v) pumped at a flow rate of 2.0 mL/min for 30 min. Enantiomer separation was carried out on a 250 mm x 4.6 mm I.D. stainless-steel silica column (Whatman Partisil 5, NJ, USA).

2.5.1.2 Stock Solutions

Separate 4000 μg/mL solutions of *rac*-preprenalterol, *rac*-prenalterol, and 2000 μg/mL *rac*-tocainide hydrochloride (internal standard) were prepared in distilled water and stored at 4°C. These were used to prepare working solutions of 4, 20, 100, 200, 300, and 400 μg/mL of *rac*-preprenalterol and *rac*-prenalterol in microbial broth. A stock solution of S-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) [0.2% v/v] was prepared in chloroform and stored under nitrogen at -10°C (solution A). A stock 2M sodium carbonate buffer was adjusted to pH 9.9 with sodium bicarbonate (solution B).

2.5.1.3 Sample Preparation

Suitable volumes of each *rac*-preprenalterol and *rac*-prenalterol working solutions were diluted with distilled water to give final concentrations of 0.4, 2, 10, 20, 30, and 40 µg/mL of each. Stock *rac*-tocainide internal standard solution (diluted 1:10) was added to each tube at a final concentration of 10 µg/mL. Aliquots of 500 µL were transferred to clean glass test tubes giving 0.2, 1.0, 5.0, 10, 15, and 20 µg of drug per tube. Solution B (150 µL) and ethyl acetate (3.0 mL) were added to each tube and these mixtures were vortex-mixed for 15 s and centrifuged at 1800 x g for 5 min. The organic layer was removed, transferred to a clean glass test tube, and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 500 µL chloroform and derivatized with 150 µl of NEIC (solution A) at room temperature. These solutions were vortexed for 10 s and evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with 200 µL of chloroform and reacted with 10 µl of ethanolamine. Aliquots ranging from 40 to 150 µL were injected onto the HPLC.

2.5.2 HPLC Method for Resolving the Enantiomers of Mexiletine and Two Major Metabolites Isolated From Microbial Fermentation Medium.

2.5.2.1 Chromatography

The HPLC system (Waters, Mississauga, Canada) consisted of a model 590 pump, 712 WISP auto sampler and 470 scanning fluorescence detector set at 280 nm and 340 nm for excitation and emission, respectively. Chromatographic data were collected using an NEC Powermate sx/16 computer (Boxborough, MA, U.S.A.). Peak integration was determined using the Baseline 810 program (Waters, Mississauga, Canada). Enantiomer separation was carried out on a 250 x 4.6 mm (I.D.) stainless steel Partisil 5 column (Phenomenex, Torrance, CA, U.S.A.). The mobile phase consisted of hexane : chloroform : methanol (65:34:1 v/v) pumped at a flow-rate of 0.8 mL/min, for 12 min, and 2.5 mL/min for the remaining 23 min.

2.5.2.2 Stock Solutions

Separate 4000 μg/mL solutions of *rac*-mexiletine hydrochloride, *rac*-hydroxymethylmexiletine (HMM) hydrochloride, *rac*-*p*-hydroxymexiletine (PHM) oxalate and *rac*-prenalterol hydrochloride (internal standard) were prepared in distilled water and stored at 4°C. These were used to prepare working solutions containing 16, 32, 64, 100, 200 and 300 μg/mL of *rac*-mexiletine, *rac*-HMM, and *rac*-PHM in microbial broth. A stock solution of NEIC (0.1% v/v) was prepared in chloroform and stored under nitrogen at -10°C (solution 1). A solution of *n*-butylamine (0.33% v/v in chloroform, solution 2) was freshly prepared prior to sample analysis

2.5.2.3 Sample Preparation

Suitable volumes of each *rac*-mexiletine, *rac*-HMM, and *rac*-PHM working solutions were diluted with distilled water to give final concentrations of 0.8, 1.6, 2.4, 5.0, 10.0, and 15.0 μg/mL of each racemate. Stock *rac*-prenalterol internal standard solution was added to each tube at a final concentration of 20 μg/mL. Aliquots of 250 μL were transferred to clean glass test tubes giving 0.2, 0.4, 0.8, 1.25, 2.5, and 3.75 μg of racemic drug per tube. Sodium carbonate (100 μL of 0.2 M solution) and diethyl ether (2.0 mL) were added to each tube and these mixtures were vortexed for 15 s and centrifuged at 1800 x g for 4 min. The organic layer was removed and transferred to a clean glass test tube. The ether extraction step was repeated and the combined extracts were evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 300 μL of chloroform and derivatized with 75 μL of NEIC (solution 1) at room temperature. These solutions were vortexed for 10 seconds and again evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with 220 µL of chloroform and reacted with 300 µL of *n*-butylamine (solution 2) to derivatize unreacted NEIC. Aliquots ranging from 10 to 65 µL were injected onto the HPLC (Freitag *et al.* 1993).

2.5.3 Reverse Phase HPLC Method for Resolving Acetylated Mexiletine, Acetylated HMM, and Acetylated PHM From Fermentation Medium.

2.5.3.1 Chromatography

The HPLC system (Waters, Mississauga, ON, Canada) consisted of a model 590 pump, 712 WISP auto sampler and 486 tunable U.V. detector set at 215 nm. Chromatographic data were collected using an NEC Powermate sx/16 computer (Boxborough, MA, U.S.A.). Peak integration was determined using the Millenium program [Ver. 2.0] (Waters, Mississauga, Canada). Seperation was carried out on a 125 x 4.6 mm (i.d.) stainless steel Partisil 5 column ODS-3 (Whatman, Clifton, NJ, USA). The mobile phase consisted of 10 mM potassium phosphate buffer (pH 4.2):methanol:acetonitrile (70:15:15 v/v) pumped at a programmed flow-rate of 0.7 mL/min, for the first 10 min, and 1.2 mL/min for the remaining 20 min.

2.5.3.2 Stock Solutions

A 4000 µg/mL solution of *rac*-acetyl-mexiletine (AMEX), 4150 µg/mL solution of *rac*-acetylHMM (AHMM), 3450 µg/mL solution of *rac*-acetyl-PHM (APHM) and 400 µg/mL solution of the internal standard *rac*-acetyltocainide (ATOC) were prepared in methanol and stored at 4°C. These were used to prepare working solutions containing 16, 32, 64, 100, 200 and 300 µg/mL of AMEX, AHMM, and APHM in microbial broth.

2.5.5.3 Sample Preparation

Suitable volumes of each *rac*-AMEX, *rac*-AHMM, and *rac*-APHM working solutions were diluted with distilled water to give final concentrations of 0.8, 1.6, 2.4, 5.0, 10.0, and 15.0 μg/mL of each compound. Stock *rac*-ATOC internal standard solution was added to each tube at a final concentration of 20 μg/mL. Aliquots of 0.25 mL were transferred to clean glass test tubes giving 0.2, 0.4, 0.8, 1.25, 2.5, and 3.75 μg of racemic drug per tube. Sulfuric acid (500 μL of 0.6 M solution) and diethyl ether (4.0 mL) were added to each tube and these mixtures were vortexed for 15 s and centrifuged at 1800 x g for 4 min. The organic layer was removed, transferred to a clean glass test tube, and evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with 220 μL of mobile phase. Aliquots ranging from 50 to 150 μL were injected onto the HPLC. Chromatograms depicting typical HPLC traces obtained from a standard curve and unknown sample can be seen in figures 2-8 and 2-9.

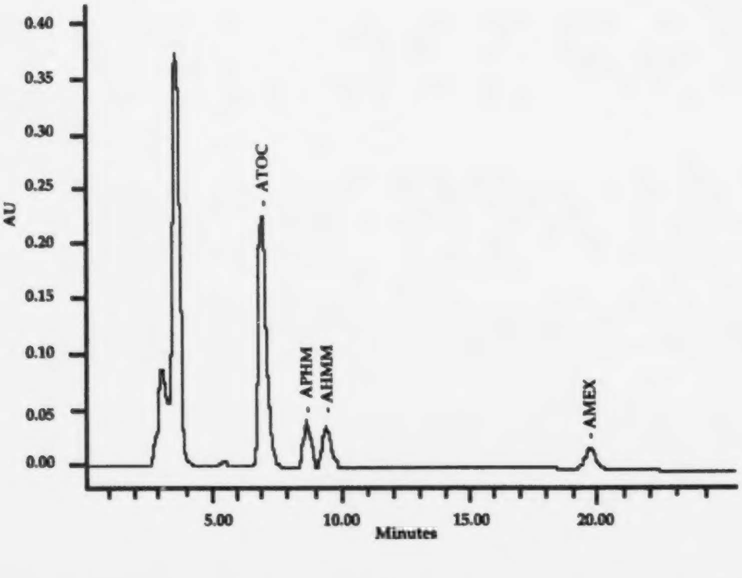


Figure 2- 8. Chromatogram of trypticase soy broth spiked with 0.40μg of *rac*-APHM, *rac*-AHMM, and *rac*-AMEX.

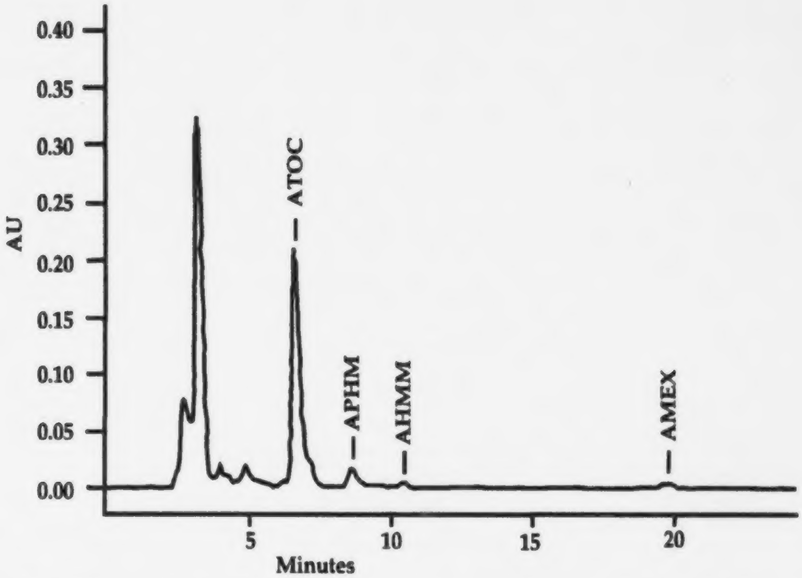


Figure 2- 9. Chromatogram of a 36 hour trypticase soy broth sample inoculated with *rac*-mexiletine.

2.5.4 HPLC Method for Resolving the Enantiomers of Propranolol and 4-Hydroxypropranolol Isolated From Microbial Fermentation Media.

2.5.4.1 Chromatography

The HPLC system (Waters, Mississauga, ON, Canada) consisted of a model 590 pump, and 712 WISP autosampler. Fluorescence detection (Applied Biosystems model 980) was set at 220 nm for excitation and 360 nm for emission. Chromatographic data were collected using an NEC Powermate sx/16 computer (Boxborough, MA, U.S.A.). Peak integration was determined using the Millenium program [Ver. 2.0] (Waters, Mississauga, ON, Canada). Enantiomer separation was carried out on a 250 x 4.6 mm (i.d.) stainless steel Partisil 5 column (Whatman, Clifton, NJ, USA). The mobile phase consisted of hexane : chloroform : methanol (85:13:2 v/v) pumped at a flow-rate of 2.0 mL/min for 20 min.

2.5.4.2 Stock Solutions

Separate 4000 μg/mL solutions of *rac*-propranolol hydrochloride, *rac*-4-hydroxypropranolol hydrochloride (4HOP) and a 8000 μg/mL solution of *rac*-pindolol (internal standard) were prepared in distilled water and stored at 4°C. These were used to prepare working solutions containing 10, 16, 32, 100, 200 and 300 μg/mL of propranolol and 4HOP in microbial broth. A stock solution of NEIC (0.1% v/v) was prepared in chloroform and stored under nitrogen at -10°C (solution 1). A solution of *n*-butylamine (0.33% v/v in chloroform, solution 2) and dimethylaminopyridine (DMAP) [0.020 μg/mL in chloroform, solution 3] were freshly prepared prior to sample analysis.

2.5.4.3 Sample Preparation

Suitable volumes of each *rac*-propranolol and *rac*-4-HOP working solutions were diluted with distilled water to give final concentrations of 0.5, 0.8, 1.6, 5.0, 10.0, and 15.0 μg/mL of each racemate. Stock *rac*-pindolol internal standard solution was added to each tube at a final concentration of 10 μg/mL. Aliquots of 250 μL were transferred to clean glass test tubes giving 0.125, 0.2, 0.4, 1.25, 2.5, and 3.75 μg of drug per tube. Sodium carbonate (0.5 M)/acetic acid buffer (pH 10.1), [100 μL] and diethyl ether (3.0 mL) were added to each tube and these mixtures were vortexed for 15 s and centrifuged at 1800 x g for 4 min. The organic layer was removed, transferred to a clean glass test tube, and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 300 μL of chloroform and derivatized with 75 μL of NEIC (solution 1) and 50 μL of DMAP (solution 3) at room temperature. These solutions were vortexed for 10 seconds and again evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with 220 μL of chloroform and reacted with 300 μL of *n*-butylamine (solution 2). Aliquots ranging from 10 to 65 µL were injected onto the HPLC. Chromatograms depicting typical HPLC traces obtained from a standard curve and unknown sample can be seen in figures 2-10 and 2-11. The different retention times seen in these two chromatograms was due to temperature fluctuations in the laboratory.

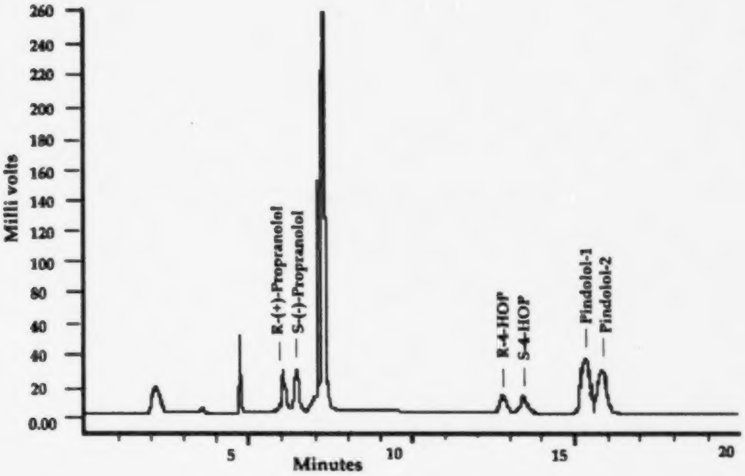


Figure 2- 10. Chromatogram of yeast extract broth spiked with 0.20µg of *rac*-propranolol and *rac*-4-HOP.

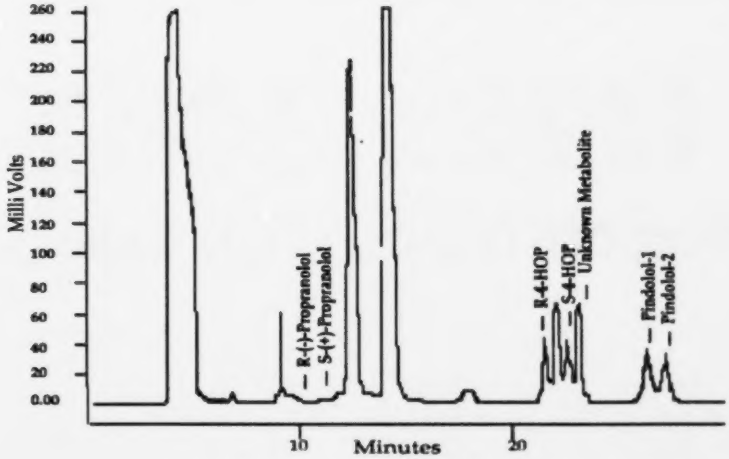


Figure 2- 11. Chromatogram of an 18 hour yeast extract broth sample inoculated with 300 µg/mL *rac*-propranolol.

Table 2- 7. Accuracy (analytical recovery) and precision (coefficient of variation) of the analytical method (n=9, 3 sets x 3 days). Coefficient of variation (C.V.) and analytical recovery (A.R.) are expressed as percentages *.

R-(+)-Propranolol precision (C.V.) and accuracy (A.R.).

Amount of Enantiomer added (μg)	R-(+)-Prop Avg (μg)	R-(+)-Prop StDev	R-(+)-Prop C.V.	R-(+)-Prop Analytical Recovery
0.0625	0.07	0.01	8.64	114.8
0.1	0.10	0.01	6.61	99.2
0.2	0.19	0.01	3.50	93.5
0.625	0.61	0.02	3.30	97.9
1.25	1.23	0.05	3.78	98.3
1.875	1.91	0.05	2.80	101.2

S-(+)-Propranolol precision (C.V.) and accuracy (A.R.).

Amount of Enantiomer added (μg)	S-(-)-. Prop Avg (μg)	S-(-)-Prop StDev	S-(-)-Prop C.V.	S-(-)-Prop Analytical Recovery
0.0625	0.07	0.01	8.97	111.6
0.1	0.10	0.01	6.12	100.0
0.2	0.19	0.00	2.31	96.4
0.625	0.62	0.02	3.33	99.4
1.25	1.21	0.07	5.37	97.0
1.875	1.90	0.04	2.07	101.4

4-HOP-1 precision (C.V.) and accuracy (A.R.).

Amount of Enantiomer added (μg)	4-HOP-1 Avg. (μg)	4-HOP-1 StDev.	4-HOP-1 C.V.	4-HOP-1 Analytical Recovery
0.0625	0.07	0.01	17.85	118.1
0.1	0.11	0.02	14.81	107.7
0.2	0.19	0.04	20.41	95.5
0.625	0.57	0.09	15.86	91.0
1.25	1.25	0.06	4.73	100.3
1.875	1.87	0.08	4.02	99.9

4-HOP-2 precision (C.V.) and accuracy (A.R.).

Amount of Enantiomer added (μg)	4-HOP-2 Avg. (μg)	4-HOP-2 StDev.	4-HOP-2 C.V.	4-HOP-2 Analytical Recovery
.025	0.05	0.01	26.94	193.7
0.0625	0.08	0.02	20.43	119.6
0.1	0.11	0.02	16.41	108.3
0.2	0.19	0.04	21.24	94.0
0.625	0.57	0.09	16.49	91.7
1.25	1.25	0.07	5.38	99.5
1.875	1.88	0.07	3.90	100.3

* Accuracy is defined as % analytical recovery which is the mean result expressed as a percentage of the amount of analyte added (found/added x 100). Precision was determined by calculating the inter-assay coefficient of variation.

2.5.5 Reverse Phase HPLC Method for Resolving *rac*-Propranolol and *rac*-4-Hydroxypropranolol Isolated From Microbial Fermentation Media.

2.5.5.1 Chromatography

The HPLC system (Waters, Mississauga, ON, Canada) consisted of a model 590 pump, 712 WISP auto sampler and 470 scanning fluorescence detector set at 215 nm and 395 nm for excitation and emission, respectively. Chromatographic data were collected using a NEC Powermate sx/16 computer (Boxborough, MA, U.S.A.). Peak integration was determined using the Millenium program [Ver. 2.0] (Waters, Mississauga, ON, Canada). Enantiomer separation was carried out on a 125 x 4.6 mm (i.d.) stainless steel Partisil 5 column ODS-3 (Whatman, Clifton, NJ, USA.). The mobile phase consisted of methanol:water (35:65 v/v) pumped at a flow-rate of 0.8 mL/min for 20 min. The mobile phase also contained 10 mL/L acetic acid and 300 µl /L of triethylamine (TEA).

2.5.4.2 Stock Solutions

Separate 2000 µg/mL solutions of *rac*-propranolol hydrochloride, *rac*-4HOP hydrochloride and a 8000 µg/mL solution of *rac*-pindolol (internal standard) were prepared in distilled water and stored at 4°C. These were used to prepare working solutions containing 4, 16, 32, 64, 100, 200 and 300 µg/mL of propranolol and 4HOP in microbial broth.

2.5.4.3 Sample Preparation

Suitable volumes of each working solution were diluted with distilled water to give final concentrations of 0.2, 0.8, 1.6, 2.4, 5.0, 10.0, and 15.0 µg/mL of each compound. Stock internal standard solution was added to each tube at a final concentration of 40 µg/mL. Aliquots of 250 µL were transferred to clean glass test tubes giving 0.05, 0.2, 0.4, 0.8, 1.25, 2.5, and 3.75 µg of drug per tube. Sodium carbonate (0.5 M)/acetic acid buffer (pH 10.1) [100 µL] and diethyl ether (3.0 mL) were added to each tube and these mixtures were vortexed for 15 s and centrifuged at 1800 xg for 4 min. The organic layer was removed, transferred to a clean glass test tube, and evaporated to dryness under a gentle stream of nitrogen.. The residue was reconstituted in 200 µL of mobile phase containg 2 g/L ascorbic acid. Aliquots ranging from 75 to 180 µL were injected onto the HPLC. Representitive chromatograms demonstrating a typical trace obtained from a standard curve and unknown sample are found in figures 2-12 and 2-13.

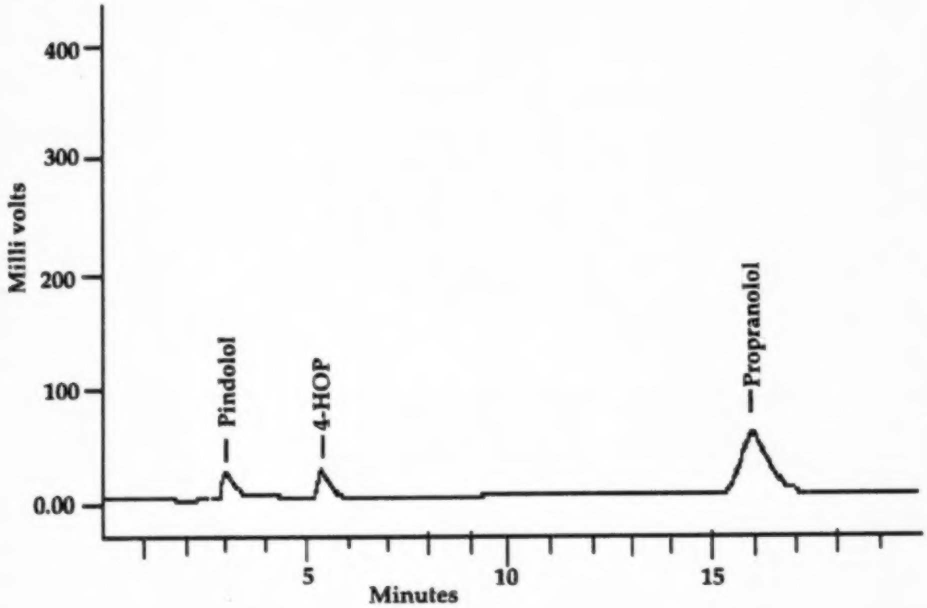


Figure 2- 12. Chromatogram of yeast extract broth spiked with 0.20µg of *rac*-propranolol and *rac*-4-HOP.

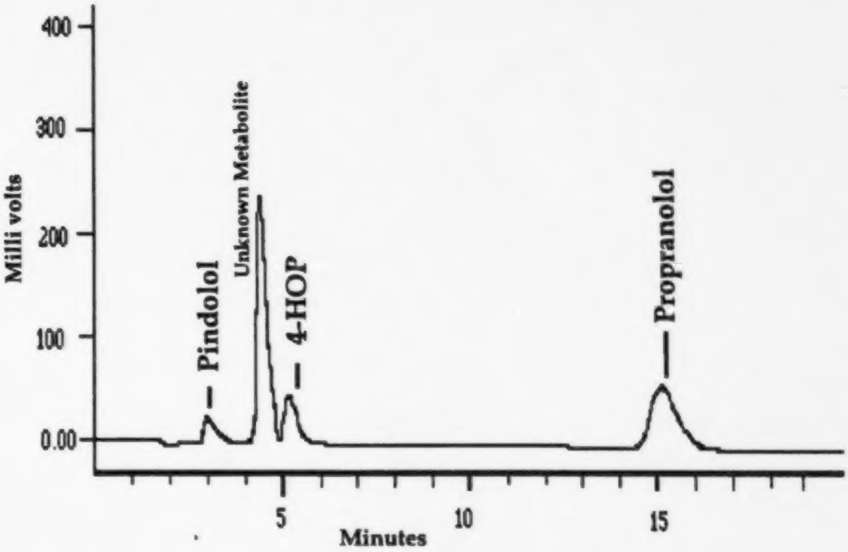


Figure 2- 13. Chromatogram of an 22 hour yeast extract broth sample inoculated with 270 μg/mL *rac*-propranolol.

Table 2- 8. Accuracy (analytical recovery) and precision (coefficient of variation) of the analytical method (n=7). Coefficient of variation (C.V.) and analytical recovery (A.R.) are expressed as percentages.

Inter-Day Variation (N=4)				
Propranolol				
Amount Added (ug)	Average Propranolol Conc. (ug)	Standard Dev. Propranolol	Coef. of Var. Propranolol	Analytical Prop Recovery (Average, %)
0.05	0.05	0.008	14.84	97.9
0.20	0.24	0.025	10.62	118.1
0.40	0.45	0.032	7.02	113.2
0.80	0.93	0.073	7.86	116.4
1.25	1.41	0.047	3.30	113.1
2.50	2.38	0.185	7.78	95.4
3.75	3.65	0.081	2.23	97.3

Table 2-8 Continued

Inter-Day Variation (N=4)				
4-HOP				
Amount Added (ug)	Average 4-HOP Conc. (ug)	Standard Dev. 4-HOP	Coef. of Var. 4-HOP	Analytical 4-HOP Recovery (Average, %)
0.05	0.04	0.004	9.80	85.2
0.20	0.21	0.009	4.01	106.4
0.40	0.46	0.048	10.56	114.7
0.80	0.83	0.041	4.99	103.6
1.25	1.40	0.061	4.37	111.5
2.50	2.45	0.161	6.57	98.1
3.75	3.64	0.241	6.63	96.9

Intra-Day Variation (N=3)				
Propranolol				
Amount Added (ug)	Average Propranolol Conc. (ug)	Standard Dev. Propranolol	Coef. of Var. Propranolol	Analytical Prop Recovery (Average, %)
0.05	0.05	0.008	14.39	98.9
0.20	0.21	0.031	15.06	109.5
0.40	0.44	0.051	11.46	110.1
0.80	0.95	0.056	5.92	118.6
1.25	1.26	0.192	15.19	100.9
2.50	2.58	0.069	2.68	102.9
3.75	3.59	0.097	2.69	95.7

Intra-Day Variation (N=3)				
4-HOP				
Amount Added (ug)	Average 4-HOP Conc. (ug)	Standard Dev. 4-HOP	Coef. of Var. 4-HOP	Analytical 4-HOP Recovery (Average, %)
0.05	0.04	0.005	12.28	86.2
0.20	0.21	0.029	13.68	102.3
0.40	0.46	0.051	10.95	115.3
0.80	0.84	0.030	3.60	105.1
1.25	1.25	0.188	15.03	99.9
2.50	2.62	0.074	2.84	104.6
3.75	3.54	0.173	4.90	94.3

Table 2- 9. Intra-day variation calculated by averaging the concentration measured, over three days, using three different amounts of drug (N=3).

Intra-Day Variation as Measured by Quality Control (Q.C.) Samples Propranolol						
Q. C. Amount of Drug Added	Day 1 Measured Conc. (μg)	Day 2 Measured Conc. (μg)	Day 3 Measured Conc. (μg)	Average Measured Conc. (μg)	Standard Deviation	Coefficient of Variation
0.20 μg	0.18	0.21	0.18	0.19	± 0.018	9.2%
0.80 μg	0.80	0.83	0.91	0.85	± 0.057	6.7%
2.50 μg	2.47	3.0	2.67	2.71	± 0.270	9.8%

Intra-Day Variation as Measured by Quality Control (Q.C.) Samples 4-HOP						
Q. C. Amount of Drug Added	Day 1 Measured Conc. (μg)	Day 2 Measured Conc. (μg)	Day 3 Measured Conc. (μg)	Average Measured Conc. (μg)	Standard Deviation	Coefficient of Variation
0.20 μg	0.22	0.22	0.18	0.20	± 0.026	12.5%
0.80 μg	0.46	0.93	0.82	0.83	± 0.094	11.3%
2.50 μg	2.6	3.0	2.63	2.75	± 0.220	7.9%

2.5.5 Experimental Procedure and Quality Control Data.

Broth samples were analyzed using separate calibration curves for each sample tray prepared (48 samples per tray). In addition, to verify results, quality control samples were included. Known quantities of drug (alternating low, medium, and high concentrations) were analyzed after every sixth sample. Results had to be within 20% of the actual amount of drug added for the preceeding unknown concentrations to be valid. One quality control sample per run was permitted to be over 20%. Quality control data are available. On account of the large amount of raw data, they are not included in this thesis.

2.6 Aqueous Acetylation of Mexiletine, Hydroxymethylmexiletine, p-Hydroxymexiletine, and Tocainide.

The procedure utilized to acetylate the primary amine functional groups of mexiletine, HMM, PHM and tocainide was that developed by Hargesheimer *et al.* (1981) for acetylation of anilines. Identity of the products was verified using electron impact mass spectral analysis. Electron impact (EI) mass spectrum, m/z (% relative abundance) data is listed below:

Acetylmexiletine: 222 (2) molecular ion, 100 (100), 58 (88).

Acetylhydroxymethylmexiletine: 237 (2) molecular ion, 100 (100), 58 (88).

Acetyl-p-hydroxymethylmexiletine: 237 (2) molecular ion, 100 (100), 58 (88).

Proposed identity of the major fragments:

$C_5H_{10}NO\rceil^{+\bullet}$

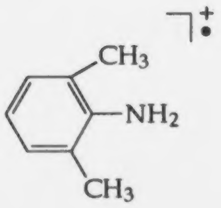
m/z : 100 (100)

$C_2H_4NO\rceil^{+\bullet}$

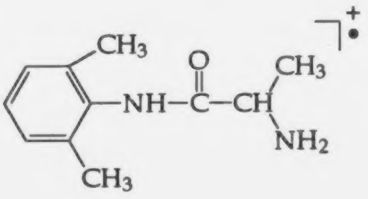
m/z : 58 (88)

Acetyltocainide: 234 (8) molecular ion, 192 (55), 121 (100).

Proposed structures of the major fragments:



m/z : 121 (100)



m/z : 192 (50)

2.7 Acid catalyzed Deuteration of the Activated Ortho and Para Positions on the Naphthyl Ring of Propranolol.

[This work was completed by a graduate student in our faculty: Kevin Morin]

Deuteraton of *rac*-propranolol, R-(-)-propranolol, and S-(+)-propranolol was performed using the following procedure. The method was identical for each enantiomer and the racemic mixture. A solution of 5% DCl/CH_3COOD in D_2O was prepared by hydrolysis of acetyl chloride (31 mg, 0.39mMol) in 2 mL of D_2O. Propranolol HCl (50 mg, 0.169 mMol) was added to the solution and the mixture was heated to 100°C for 30 min. The reaction mixture was cooled to room temperature, neutralized and made alkaline with NH_4OH. The mixture was then extracted with diethyl ether (3 x 10 mL) and the organic extract was dried over sodium sulfate. The solvent was evaporated and the residue recrystalized using methanol/H_2O. The yields of recovered material were 83-92%. ^1H-nmr indicated that 2 atoms of deuterium were qualitatively introduced into the molecule. The H-3 proton resonance triplet at 7.38 in the unlabelled propranolol spectrum collapsed to a singlet at this frequency providing evidence that the deuterium atoms were incorporated in the 2 and 4 positions on the naphthyl ring. High resolution mass spectrometry indicated that the labeling occurred quantitatively with > 91% labelling efficiency for each compound. Enantiomeric purity was confirmed by HPLC after derivatization using S-(+)-NEIC. Retention times were compared to authentic unlabeled materials using identical procedures.

3.0 Fungal Growth

3.1 Introduction

Fungi are a diverse group of heterotrophic microorganisms constituting a separate kingdom within which an estimated 250,000 species exist (Hawksworth *et al.* 1983). A descriptive list identifying a few of these would include the molds found on decaying matter, mushrooms, puffballs, mildews, slime molds, smuts, and rusts. They are comprised of cells termed eucaryotic as they possess a nucleus, nuclear membrane, and mitochondria. Therefore, on a subcellular level they resemble plants and animals. A unique characteristic of fungi is the manner in which they grow and obtain nutrients. When a suitable food source is encountered, hydrolytic enzymes are released to digest the food externally. Products formed are transported into the cell to sustain this system of feeding threads which rapidly spread to engulf the food source. This method of growth helps to decrease competition from other organisms. Fungi display many other characteristics that have led to their placement in a separate kingdom which will not be listed here. Instead, the discussion will focus on traits that impact on biotransformation experiments starting with a more detailed explanation of growth.

Filamentous fungi grow as a branched series of tubular filaments (hyphae) which are collectively called mycelia. The individual hyphae are surrounded by a rigid cell wall that, in the majority of cases, will contain chitin. However, cellulose and chitosan have also been identified as important structural components (Wessels *et al.* 1990). Their growth is initiated from resting spores which have low levels of metabolic activity (Gottlieb 1978). When a suitable food source is provided metabolism increases rapidly followed by spore enlargement (swelling) and the emergence of a germ tube(s) (Trinci 1971). As the germ tube grows it branches repeatedly forming filaments with numerous hyphal tips. It is important to emphasize that in the majority of cases, fungal growth occurs strictly at the hyphal (apical) tip. Filamentous fungi grow by continually extending their length (Jackson *et al.* 1993) and not by increasing in number. Once growth is well established, specific environmental conditions will encourage the formation of reproductive mycelia. These specialized structures generate resting spores from which fungal growth can be started again. This brief overview of their life cycle describes growth at the microscopic level only. An interesting characteristic of filamentous fungi is that they display a macroscopic morphology which is influenced by environmental conditions.

When bacterial growth is examined in stationary liquid culture the individual cells become evenly distributed throughout the media. In this environment, all growing cells have equal access to nutrients dissolved in solution. Under identical conditions, spores from filamentous fungi will not disperse throughout a stationary liquid medium. Their hydrophobic nature encourages development of interconnecting mats of floating mycelium where the upper surface is exposed to the atmosphere (aerobic growth) while the lower surface absorbs nutrients (Burkholder *et al.* 1945). Growth on solid media is similar as hyphae spread in a radial fashion, from the inoculum, above and below the agar surface (Trinci 1971b).

If agitation is applied to spores inoculated into liquid media, this pattern of development changes dramatically. By employing shaken (submerged) culture technique, fungal morphology can range from individual pellets, comprised of compact masses of hyphae, to filamentous mycelia that disperses evenly throughout a medium (Whitaker and Long 1973, Reichl and Gilles 1991). There are a number of review articles which examine the unique characteristics of submerged fungal growth in more detail (Metz and Kossen 1977, Suijdam *et al.* 1980, Royer and Rouleau 1985, Cox and Thomas 1991). The macroscopic form of growth will be determined by an organism's genetic makeup and culture conditions.

We were interested in this aspect of growth because of studies correlating morphology with specific fungal activities. For example, a pelleted form of *Aspergillus niger* is essential for the enzyme polygalacturonase to be expressed (Hermersdorfer *et al.* 1987). When *Penicillium chrysogenum* is grown as mycelial fragments, penicillin production improves (Smith *et al.* 1990). In addition, fungal morphology can physically alter growth by affecting oxygen transfer, nutrient consumption, and media viscosity. Therefore, when using fungi in submerged culture for biotransformation experiments it was important to establish parameters which gave rise to a consistent morphology. A consequence of predictable growth was an ability to optimize metabolite production.

3.2 Phase I Fungal Growth

A two phase submerged culture technique was employed to study fungal biotransformation reactions. Phase I cultures were designed to produce a large biomass of rapidly growing cells. Phase II cultures, prepared from phase I mycelia, were utilized to carry out metabolic studies. To initiate phase I growth, spores were harvested from the surface of a maintenance slant to which 20 mL of sterile water had been added. The spore suspension thus generated was transferred into a 125 mL flask containing 25 mL of medium (Dombroski 1988). This method of initiating phase I growth was only used for a brief period of time. The outcome was unpredictable, yielding poor growth that varied in morphology and quantity between experiments. It therefore became important to start a process for determining conditions that would consistently give rise to specific types and amounts of growth (Figures 3-2B, 3-2C, 3-2D). We began by examining media formulations used by other laboratories (Table 3-L). Dombroski's protocol (1988) recommended the use of a minimal medium called czapek dox broth (Figure 3-2C). This contained sodium nitrate (3 g/L) and sucrose (30 g/L) as the nitrogen and carbon source respectively. Other recipes employed similar carbon sources but incorporated more complex forms of organic nitrogen such as yeast extract. A disadvantage of using undefined nitrogen sources was the possibility of extracting unknown media components that could co-elute with the drug and metabolites of interest during analysis. Therefore, an experiment was designed to test different media formulations in order to find one that contained the minimum number of components necessary to generate rapid growth. The media formulations tested and their outcomes are listed in table 3-2. The organism investigated was *C. echinulata* 4145.

Table 3- 1. Media formulations used by us and other laboratories.

Foster *et al.* 1988		Cerniglia *et al.* 1990	
- Trypticase soy broth	30.0 g/L	- Sabouraud dextrose broth	30 g/L
- Yeast extract	7.0 g/L	pH 5.6	
- Glucose	16.0 g/L		
pH 7.3			
Hartman *et al.* 1990		**Reddy *et al.* 1990**	
- Pharmamedia	10.0 g/L	- Soybean meal	5.0 g/L
- Yeast extract	5.0 g/L	- Yeast extract	5.0 g/L
- Sodium chloride	5.0 g/L	- Sodium chloride	5.0 g/L
- Potassium phosphate	5.0 g/L	- Potassium phosphate	5.0 g/L
- Glucose	20.0 g/L	- Glucose	20.0 g/L
pH 7.0		pH 7.0	
Components of Czapec Dox		**Components of Trypticase Soy**	
- Saccharose	30.0 g/L	- Pancreatic digest of casein	17.0 g/L
- Sodium nitrate	3.0 g/L	- Papaic digest soybean meal	3.0 g/L
- Dipotassium phosphate	1.0 g/L	- Sodium chloride	5.0 g/L
- Magnesium sulfate	0.5 g/L	- Dipotassium phosphate	2.5 g/L
- Potassium chloride	0.5 g/L	- Dextrose	2.5 g/L
- Magnesium sulfate	0.01 g/L	pH 7.3	
pH 7.3			

This experiment demonstrated that the type of sugar used (media #4 and #5) was not a factor in generating rapid growth. However, we did not investigate its influence on the pelleting process. In addition, it appeared that a key ingredient to rapid fungal development was the addition of a complex nitrogen source like yeast extract or soy meal. These components promoted growth by providing preformed organic molecules that can be incorporated directly into a microorganism's framework. Based on this experiment, the medium formulation chosen to carry out biotransformation reactions was one that contained 20 g/L sucrose, 8.0 g/L yeast extract, 4.6 g/L sodium chloride, and potassium phosphate (monobasic and dibasic) buffer (pH 7.0). On average this medium would generate between 6.0 and 9.0 g (wet weight) of mycelia per 125 mL flask in 36 hours.

Table 3- 2. Media formulations tested and their effect on the growth of *C. echinulata* 4145 (czapec dox broth contains 30.0 g/L sucrose).

Medium # 1	Medium # 2	Medium # 3
- Czapek dox broth 35 g/L	Trypticase soy broth 35 g/L	- Czapek dox broth 35 g/L Replace sucrose with 30 g/L glucose.
+ (pellets)	+++ (1 large pellet ≈ 1" long)	+ (pellets)

Medium # 4	Medium # 5	Medium # 6
- Czapek dox broth 35 g/L Replace sucrose with 30 g/L glucose. - Yeast extract 7.6 g/L	- Czapek dox broth 35 g/L - Yeast extract 7.6 g/L	- Trypticase soy broth 35 g/L - Yeast extract 7 g/L - Dextrose 16 g/L
+++ (pellets)	+++ (1 large pellet ≈ 1" long)	+++ (pellets)

+ - poor growth
++ - good growth
+++ - excellent growth

3.2.1 Fungal Pelleting

When C. *echinulata* was grown using a yeast extract medium, the fungus developed as either discrete pellets (Figure 3-2A, 3-2B) or as a single mycelial ball (Figure 3-2D). The exact morphology obtained was unpredictable using methods previously discussed. This problem had to be addressed to determine which pattern of growth suited our work and how it could be achieved consistently. It was hypothesized that pelleted growth would favor xenobiotic metabolism because a high surface to volume ratio would increase contact between mycelia and the drug. Initial attempts to achieve reproducible pelleted growth focused on the flask's position in the environmental shaker. Previously, cultures were incubated horizontally on the shaker's platform (Figure 3-1) circulating the medium in a laminar fashion. Using a steel bracket, flasks were tilted to an angle of 20° (Foster 1982) creating turbulent flow. Increasing agitation helped prevent individual pellets from congealing into one large mycelial ball.

Laminar flow
Turbulent flow

125 ml Erlenmeyer flask laying flat on the shaker platform.

125 ml Erlenmeyer flask at a 20° angle on the shaker platform.

Figure 3- 1. Repositioning a flask on the shaker platform to prevent laminar flow and create turbulent flow.

Another measure utilized to standardize growth was to chemically treat the flask's interior with a siliconizing [Aquasil (octadecyltrialkoxysilane)] agent (Foster 1982). This procedure was required to combat the fungus's ability to attach its mycelia to the glass at the air/medium interface. The resulting thick ring of mycelia disrupted submerged growth. Coating the flask's interior prevented attachment, forcing the fungus into solution. This created conditions favoring pellet formation.

Figure 3- 2. The varied morphologies displayed by *Cunninghamella echinulata* 4145 when grown using selected growth media.

A. Pellets of *Cunninghamella echinulata* 4145 magnified 400 times. These pellets were removed from a phase I culture after 36 hours of growth on yeast extract broth and stained.

B. *Cunninghamella echinulata* 4145 pellets harvested from a phase I culture after 36 hours of growth on yeast extract broth. The culture was diluted 1:4, with water, to provide better resolution of the individual pellets.

C. A 36 hour phase I culture of *Cunninghamella echinulata* 4145 grown in czapec dox broth.

D. *Cunninghamella echinulata* 4145 growing as a single mycelial ball.

A

B

C

D

Inoculum size was another factor considered in attempts to control fungal growth. The number of spores utilized should be sufficient to develop into a heavy suspension of growth (Whitaker and Long 1973). If the number of spores germinating is small, the lack of competition for nutrients will encourage development of a few large colonies of mycelia (Figure 3-2D). With mass inoculum all hyphae develop simultaneously provided shaking is vigorous (turbulent) enough to prevent agglomeration. In addition, competition for available nutrients will be such that each center of growth (pellet) will consume a small part of the total. This will encourage even growth throughout the media (Figure 3-2A and B).

Inoculum size was standardized by adapting a hemocytometer to count the number of spores being introduced to each flask (Section 2.3.2). This technique had an unexpected benefit by revealing that the number of spores within individual maintenance slants was variable. This was a contributing factor to the unpredictability of our previous results. Even though a spore count provides no information regarding cell viability, an inoculum of 48,000 spores/mL (for a 125 mL flask) would consistently provide pellet growth. In addition, increasing the spore concentration beyond this did not significantly increase the amount of biomass (Table 3-3, Figure 3-3).

Table 3- 3. Determining culture wet weight (grams) using different spores concentrations for an inoculum.

Inoculum Size	Culture Wet Weight (g) at Sample Time (hours)						
	2 hours	4 hours	7.5 hours	10 hours	12 hours	23 hours	36 hours
8000 spores/mL	0.15 g	0.18 g	0.25 g	0.33 g	0.40 g	2.19 g	4.71 g
	0.17 g	0.19 g	0.27 g	0.32 g	0.41 g	2.43 g	5.01 g
	0.13 g	0.15 g	0.27 g	0.35 g	0.40 g	2.11 g	4.55 g
40,000 spores/mL	0.18 g	0.20 g	0.38 g	0.51 g	0.57 g	2.88 g	7.11 g
	0.19 g	0.21 g	0.40 g	0.66 g	0.74 g	3.42 g	8.28 g
	0.19 g	0.21 g	0.38 g	0.48 g	0.62 g	3.92 g	7.87 g
104,000 spores/mL	0.20 g	0.23 g	0.68 g	1.13 g	1.32 g	5.52 g	8.55 g
	0.22 g	0.23 g	0.69 g	1.16 g	1.23 g	5.05 g	8.71 g
	0.23 g	0.25 g	0.70 g	0.92 g	1.30 g	4.96 g	8.22 g

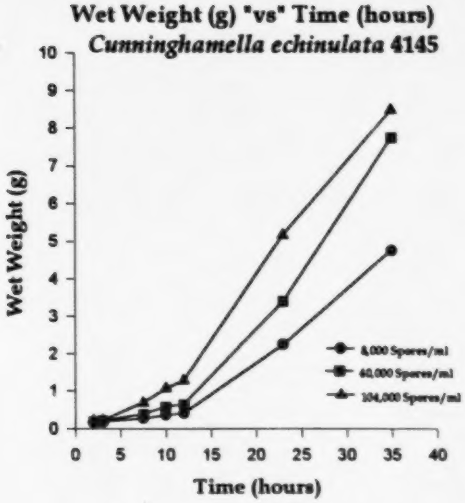


Figure 3- 3. Graphical representation of the increase in wet weight of phase I cultures using three spore concentrations as inoculum.

To summarize, various factors were critical for consistent phase I growth. A complex nitrogen source was crucial for rapid generation of large amounts of biomass. However, the choice of carbon source (glucose, sucrose) did not affect the amount of growth. In addition, it was important to utilize a spore inoculum large enough to provide competition for nutrients between individual colonies. Therefore, an inoculum of 48,000 spores/mL (for 125 mL flask) was utilized because it provided consistent pelleted growth for experiments performed. Finally, coating the flask's interior surface (Aquasil) and providing turbulent flow (angle brackets) helped create an environment that, in conjunction with a complex nitrogen source and sufficient inoculum, encouraged consistent pellet formation.

3.3 Phase II Fungal Growth

Phase I growth was designed to generate an actively growing culture to be utilized for biotransformation experiments. However, another interesting characteristic of fungi is their ability to produce large amounts of biomass in a short period of time. This exhausts a medium of important nutrients and, with an accumulation of waste products, inhibits further growth. Therefore, the mycelium was transferred into a more favorable environment for *in vitro* drug metabolism studies. Adding another step, designated as phase II growth, created a few practical problems. For example, it was difficult to find pipettes, with large enough tip openings, to transfer mycelia from one container to another. If bacteria were utilized for these experiments, their small size would make this a simple procedure. Portions of a phase I culture could be pipetted into new flasks serving as the inoculum for phase II growth. The cells would continue to grow, by binary fission, as they did in phase I. When this was attempted with *C. echinulata*, the transferred pellets grew into much larger solid pellets (2 cm in length when squashed under a microscope slide). In addition, phase II pellets did not grow as fast, or as well, as did phase I pellets.

A technique used by Dombroski (1988) and Foster (1982) to promote rapid phase II growth involved homogenizing a mature culture into a mycelial suspension. The hyphal tips generated were utilized as an inoculum (10% of phase II volume). This procedure was based on the knowledge that fungi grow strictly at their apical ends. Therefore, each hyphal tip generated had the potential of growing into an individual mycelial colony. When this protocol was attempted using a Waring blender with a 110 mL sample container the outcome was unsatisfactory*. In addition, it was difficult to maintain sterile conditions.

To avoid these problems, while taking advantage of the biomass generated in phase I, a technique used in experiments with bacteria was employed. If a bacterial culture stopped growing due to the depletion of a critical nutrient it could be centrifuged into a small pellet of cells. The spent medium would be poured off, new medium added, and growth would continue without a loss of biomass. With modifications, this protocol was adapted to biotransformation experiments. The mycelia generated in phase I was centrifuged, washed, and transferred into fresh medium where it was utilized for *in vitro* drug metabolism experiments. This procedure created an environment allowing C. *echinulata* 4145 to metabolize the test compound, *rac*-preprenalterol (Figure 3-5), to its corresponding p-hydroxy metabolite, prenalterol (86% yield). *rac*-Preprenalterol was chosen to test the new procedure because its metabolic profile was previously determined in our laboratory (Pasutto *et al.* 1985). Therefore, based on our results, we concluded this method of initiating phase II growth did not alter the established profile (Figure 3-4). In addition, utilizing all (7.7 g) or half (3.9g) the biomass from phase I produced the same amount of metabolite.

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* The homogenization procedure was repeated by a summer student, using a smaller sealed homogenizer cup from the Microbiology department. A 10% inoculum, from a mycelial suspension generated using this equipment, would give rise to pelletted phase II growth.

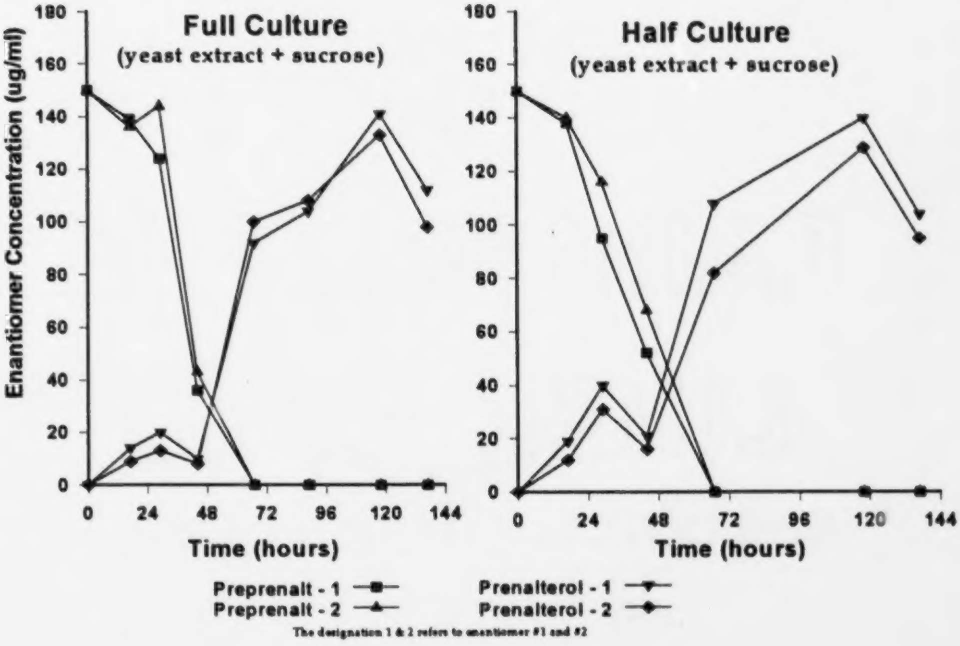


Figure 3- 4. Metabolism of Preprenalterol by all (7.7 g) and half (3.9 g) of a phase I culture of C. *echinulata* 4145 grown using a sucrose and yeast extract media.

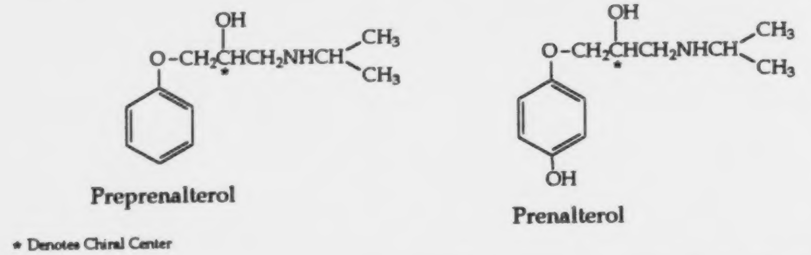


Figure 3-5. The structures of *rac*-1-isopropylamino-3-phenoxy-propan-2-ol (*rac*-preprenalterol) and the resulting product from biotransformation *rac*-prenalterol.

3.4 Evaluating Mycelial Growth

Evaluating fungal development was one aspect of this project that was examined in more detail. There was a lack of data relating an organism's growth with its drug metabolizing ability. By learning more about an organism's growth characteristics, the information gathered could be utilized to optimize metabolite formation. However, evaluating fungal growth was a difficult endeavor because of the special nature of mycelia. Fungi develop by extending their apical tips which change in composition with age. At any given time, parts of a culture might be growing while another is aging or dying. In addition, fungi may store reserve substances such that an increase in weight can occur without the formation of new hyphae. Therefore, finding a reliable method for evaluating growth was not an easy task. Our goal was to develop a simple, reliable procedure providing a window through which fungal activity could be viewed during a biotransformation experiment.

3.4.1 Weight Determination

One method of estimating mycelial growth was to determine its mass by weighing specified volumes of mycelia, or an entire culture, at specified time intervals. This would provide information pertaining to an organism's increase in weight over time. It is important to emphasize that this procedure cannot distinguish between growth (synthesis of new protein) and proliferation (increase in weight). However, both processes will be referred to as growth. By relating metabolite production to an increase in wet weight, it was possible to determine when an organism was capable of processing a drug. In addition, it was feasible to ascertain whether growth was necessary for drug biotransformations.

Wet weight determination was the first attempt made to evaluate phase I mycelial growth. This was accomplished by repeatedly harvesting the contents (25 mL) of a 125 mL Erlenmeyer flask at specified intervals. The culture's mass was determined by centrifuging the flask's contents at 900 x g for 5 min, removing the medium, weighing the pellet (wet weight), and transferring the mycelia plus liquid back into the flask from which it was removed. This provided information regarding the rate of growth and the culture's condition

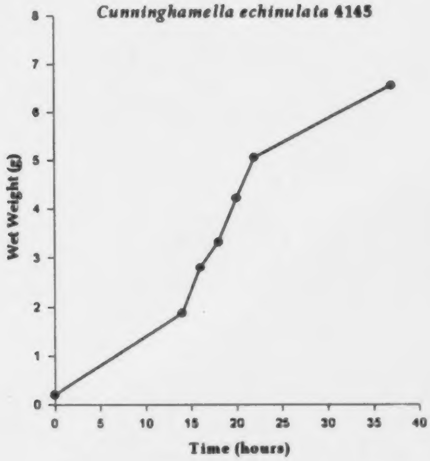


Figure 3- 6. Wet weight of the mycelia contained in a flask inoculated with 30,000 spores/mL grown using 25 mL of yeast extract and sucrose media.

when utilized for phase II experiments. Results from this study can be seen in figures 3-4 and 3-6. Both graphs illustrate that, when using spores to initiate phase I growth, there was a lag period of approximately 12 hours followed by a rapid increase in mass. In addition, 36 hour phase I mycelia were still increasing in size. Therefore, phase I experiments provided a large biomass of growing mycelia for the phase II biotransformation experiments.

3.4.2 Glucose Utilization

Finding a direct method to routinely evaluate mycelial growth proved to be difficult, requiring a consideration of indirect methods. An example of an indirect procedure is measuring bacterial growth using absorbance. In solution, bacteria form a suspension of fine particles, with a considerable degree of transparency, allowing one to estimate their number per unit volume with reasonable accuracy. However, this was not appropriate for mycelial cultures because fungal growth rapidly becomes thick and full of clumps creating conditions unsatisfactory for light transmission.

Using chemical analysis, to monitor key media components, is another indirect method of evaluating growth. When a fungal culture is grown in a closed system, its weight will be proportional to the quantity of nutrients consumed. Monitoring uptake of a specific ingredient, like glucose, can provide useful information about how an organism grows. However, if glucose is used as an indicator, it is important to realize this sugar can be converted into substances like gluconic acid. Therefore, large amounts of glucose can disappear from a medium without a corresponding increase in cell weight. This must be taken into consideration when interpreting experimental data. An advantage of using glucose analysis to follow fungal growth, was its simplicity which helped with routine testing. In addition, there are numerous enzymatic (colorimetric) methods, including the use of Sigma's Trinder reagent kit, which are available commercially. Results of a study monitoring phase I and II glucose concentration for the fungus *C. echinulata* 4145, can be seen in figures 3-7 and 3-8.

An interesting feature of the phase I data was its correlation with the pattern of growth determined using wet weight. Both methods demonstrated that, with an inoculum of 40,000 spores/mL, *C. echinulata* 4145 went through a lag period of growth lasting at least 12 hours. This was followed by a rapid increase in weight which matches the decrease in glucose concentration (log phase of growth). In addition, glucose supplies were exhausted after 22 hours, matching the time when increases in weight declined. Therefore, by transferring a culture to phase II glucose is being replenished creating an environment where hyphae can continue to grow. This graph also showed an increase in glucose concentration over the first four hours which was observed in the control.

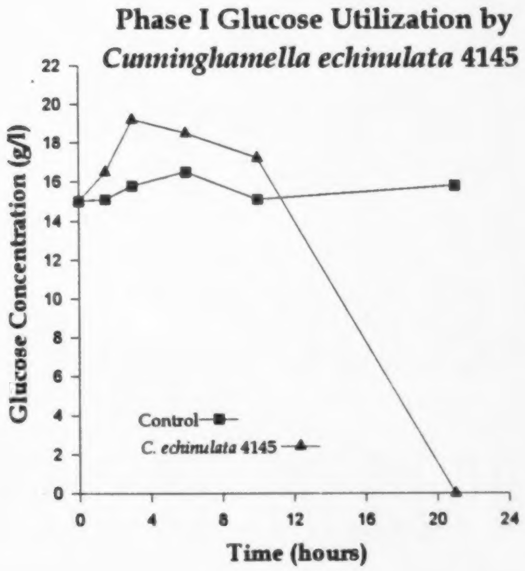


Figure 3- 7. Measuring glucose disappearance, during phase I growth of *C. echinulata* 4145, using Sigma's glucose Trinder reagent kit.

Phase I wet weight and glucose utilization data suggest that after 36 hours, fungal growth decreases due to the depletion of the carbon source. When *C. echinulata* 4145 was introduced into fresh medium, glucose concentration data shows a pattern of growth similar to the one seen in phase I. The sugar concentration decreases from 35.0 g/L to 27.0 g/L in the 30 hours, corresponding to a lag phase, followed by a rapid decrease to 4.0 g/L over the next 20 hours, resembling log growth. It was difficult to make assumptions regarding fungal growth from this information. However, it did tell us that the fungus was metabolically active during a biotransformation experiment when this protocol was used.

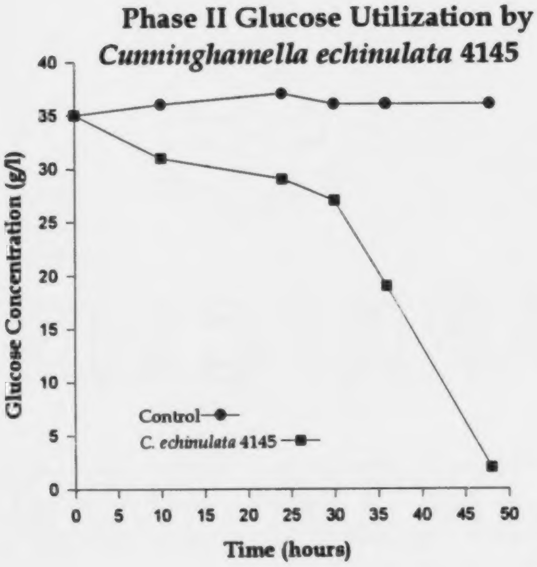


Figure 3- 8. Measuring glucose disappearance during phase II growth of *C. echinulata* 4145, using Sigma's glucose Trinder reagent kit.

Data from these graphs (Fig. 3-7, 3-8) can be correlated with preprenalterol disappearance and prenalterol formation (Fig. 3-4). The concentration of preprenalterol decreases from 150 μg/mL to 130 μg/mL in the first 30 hours followed by its complete removal over the next 42 hours. Appearance of the metabolite, prenalterol, occurs slowly over the first 40 hours followed by a dramatic increase in its production over the next 50 hours. Therefore, drug disappearance appeared to follow the pattern of glucose utilization while metabolite formation was slightly delayed. Overall, this data suggests that fungal growth is an important part of metabolite production. Data corroborating this finding will be presented in figure 4-8. When C. *echinulata* 4145 was incubated in phosphate buffer for phase II, its capacity to produce the oxidative metabolites HMM and PHM was reduced by one half.

3.5 Cytochrome P_{450} Determination

The isolation and purification of cytochrome P_{450} by Omura and Sato (1964) changed the manner in which drug metabolism could be studied. Their work made it possible to scrutinize these oxidative enzymes directly. The techniques were used to determine substrates, study reaction mechanisms, and predict possible *in vivo* metabolites (Lu and West 1978). From these investigations came the discovery of distinct forms of cytochrome P_{450} possessing specific substrate capabilities. With the advent of molecular biology, these specificities were identified at the amino acid and gene level (Gonzalez 1990). Today, isoforms of cytochrome P_{450} are being expressed in yeast cells (Truan *et al.* 1993), mammalian cells (Gonzalez *et al.* 1991), and *E. coli* (Fisher *et al.* 1992). In addition, mammalian cell lines expressing a single enzyme are commercially available.

This work also impacts the development of fungal models for studying mammalian drug metabolism. An important question that required answering was why fungi were capable of oxidizing drugs in a fashion similar to humans. In 1971, Auret *et al.,* discovered that *Cunninghamella bainieri* oxidized alkylbenzenes and that this reaction involved what is now known as an NIH shift. This mechanism of incorporating molecular oxygen into a substrate was discovered using mammalian cytochrome P_{450} (Daly *et al.* 1968) and provided indirect evidence for the existence of a similar enzyme(s) in fungi. In 1976, Ferris *et al.* isolated cytochrome P_{450} from *Cunninghamella bainieri* and found that this enzyme was capable of oxidizing benzo[a]pyrene. Gibson *et al.* (1984) built on this by using microsomes stored at -20°C from *Cunninghamella bainieri* to study N-demethylation of codeine. In addition, this enzyme was isolated from other fungi including *Candida tropicalis* (Kapelli *et al.* 1983), *Rhizopus nigricans* (Breskuvar *et al.* 1986), and *Aspergillus niger* (Reddy and Vaidyanathan 1975). Based on this work, we thought it would be feasible to isolate and quantify cytochrome P_{450} from *C. echinulata* 4145. Our rational was that if the enzyme responsible for oxidative biotransformation was cytochrome P_{450}, it would be advantageous to define growth conditions that maximized its concentration within the cell. Enhanced protein levels should correlate with increased product (metabolite) formation. In addition, evidence existed that fungal cytochrome P_{450} could utilize peroxides for substrate oxidation (Carelli *et al.* 1992). Therefore, because of our ability to produce large amounts of biomass in short periods of time, we were confident that we could isolate the enzyme in quantities sufficient for immobilization (Mazid 1993). This technique was used to entrap rat liver microsomes into alginate beads, as a method for producing glucuronide, as well as oxidative, metabolites from drugs (Haumont 1991). The advantage of this system was an ability to biosynthesize metabolites as long as the enzyme remained active.

The application of these ideas to fungal biotransformations hinged on our ability to isolate an active cytochrome P_{450} complex from the fungus *C. echinulata* 4145. However, initial attempts to purify this enzyme and generate a difference spectrum using Cerniglia's method was unsuccessful. It appeared from our result that either the protocol was not capable of isolating cytochrome P_{450}, or this enzyme did not exist in *C. echinulata* 4145. We found that regardless of the isolation procedure used, there was always an absorption maximum between

420 and 430 nm. An absorbance maximum at 450 nm was never demonstrated (Figure 3-9).

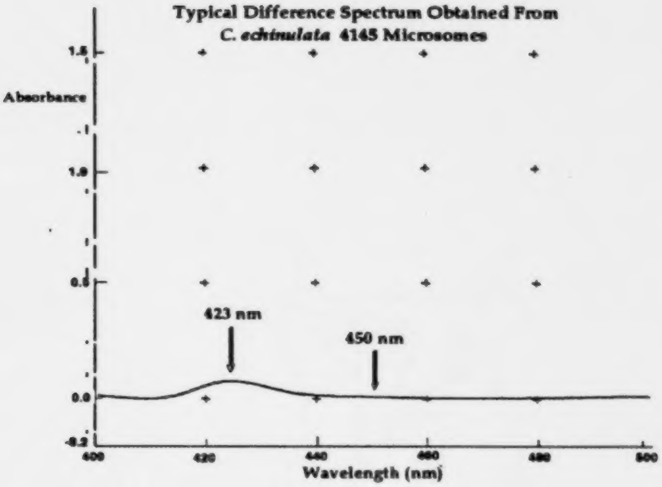


Figure 3- 9. Difference spectrum generated, using a microsomal preparation from C. *echinulata* 4145, representative of experiments performed.

One explanation for our inability to isolate an active enzyme was that the procedure could have caused a denaturing of the protein. When mammalian cytochrome P_{450} is inactivated, it displays a strong absorbance at 420 nm (Omura and Sato 1964). This peak was observed in the difference spectrum obtained from our rat liver microsome preparation (Figure 2-7). Another concern was improper grinding of the mycelia. In laboratories where cytochrome P_{450} is successfully isolated from *Cunninghamella,* a bead mill cooled with carbon dioxide was used to pulverize the cell wall (Gibson *et al.* 1984b). Mycelial resistance to mechanical disruption raised the possibility that our grinding methods were not aggressive enough to liberate the enzyme complex.

An interesting feature of cytochrome P_{450} was its requirement of a lipid component for activity, emphasizing its hydrophobic nature. This makes it difficult to solubilize once removed from the cytoplasm of fungi or plants.

Therefore, many laboratories report the use of a detergent to isolate and resuspend an active form (Kochs and Grisebach 1989). However, detergents must be used carefully as too little would be insufficient to solubilize an enzyme while too much would denature it. Numerous agents were examined (Triton X-100, Tween 80, Sodium deoxycholate), at various concentrations, and the effect was a shift in absorption from 423 nm to a maximum of 430 nm. A detergent used successfully by other laboratories to solubilize cytochrome P_{450} was Emulgen 911 . However, we could not acquire this agent as it was only available in Japan.

Currently, another active area of cytochrome P_{450} research is its characterization, and isolation, from plants (Durst *et al.* 1992, Sandermann 1992, Donaldson and Luster 1991). Interest originated from the enzyme's role in a plant's defense mechanism against pathogenic fungi and pesticides (Hallahan *et al.* 1993). An interesting theme running through this literature was the difficulty associated with isolating an active form of plant cytochrome P_{450}. A problem discussed was the difficulty encountered with solubilization. However, using Emulgen 911 as the detergent appeared to be an effective solution (Rodgers *et al.* 1993). In addition, it was demonstrated that strong absorption at 420 nm was caused by peroxidase enzymes co-purifying with cytochrome P_{450}. They generated an absorbance of 450 nm using salt (KCl) washes of the microsomes to remove the interference. We repeated this procedure using *C. echinulata* 4145 microsomes. Nevertheless, our results maintained the strong absorbtion at 430 nm.

What was originally intended to be a simple experiment turned into a valuable lesson regarding the nature of science. Research is never as easy as it appears. Even though published procedures were followed exactly, we were unable to generate an absorbtion at 450 nm when testing microsomes prepared from *C. echinulata* 4145. However, we felt the enzyme was present because this fungus was capable of producing oxidative metabolites from the substrates *rac-*

preprenalt, *rac*-mexiletine, and *rac*-propranolol. Therefore, by employing some of the newer techniques used with plants (Emulgen 911), it should be possible to generate a spectrum similar to the one obtained with rat liver microsomes (Figure 2-7). In addition, this active enzyme complex could be used for oxidative chemical reactions.

3.5.1 Enzyme Activity Testing

Even though we were unable to detect an absorbtion at 450 nm, it was possible the enzyme was present in undetectable amounts. To quantify low concentrations of cytochrome P_{450}, indirect methods are commercially available. One such test is the O-deethylation of ethoxyresorufin (Burke and Mayer 1975). However, we had already developed a sensitive HPLC assay to follow the conversion of *rac*-mexiletine into the oxidative metabolites *rac*-HMM and *rac*-PHM. Therefore, an experiment was designed to test fungal microsomes for oxidative enzyme activity. However, no activity was detected. Therefore, we concluded that the procedure used to isolate cytochrome P_{450} from *C. echinulata* 4145 was denaturing the protein.

3.6 Enzyme Induction

In general, microorganisms have tight control over transcription and translation, providing them with the ability to synthesize a particular protein in response to environmental stimuli. This characteristic has been used by industrial microbiologists to exploit control mechanisms. It has been possible to encourage microorganisms to overproduce compounds of commercial importance (e.g. citric acid production by the fungus *Aspergillus niger*). Wiedmann *et al.* (1988) used this approach to demonstrate that oxygen limitation induced the synthesis of cytochrome P_{450} mRNA in *Candida maltosa.* We wanted to determine if cytochrome P_{450} levels were affected in a similar manner using *C.*

echinulata 4145. However, as we were unable to isolate and quantify the enzyme directly, an indirect method was used to determine cytochrome P_{450} levels.

The metabolic profile of *rac*-preprenalterol, using *C. echinulata* 4145, had been previously determined in our laboratory (Pasutto *et al.* 1985). Therefore, it was used to reveal the effect of decreased oxygen concentrations on the oxidative metabolism of *rac*-preprenalterol. The results can be seen in figure 3-12.

By comparing this data with figure 3-4, it can be concluded that decreasing oxygen concentration does not induce cytochrome P_{450} activity. The result was a decrease in the rate of *rac*-prenalterol formation. Even though an increase in the rate of metabolite production was not demonstrated, we found that *C. echinulata* 4145 can grow at low oxygen concentrations (1 part oxygen was added with 99 parts nitrogen). This capability could be exploited if a metabolite sensitive to oxidation were being biosynthesized.

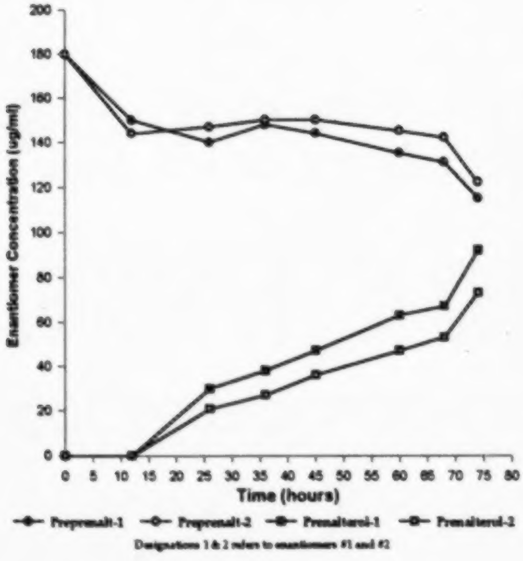


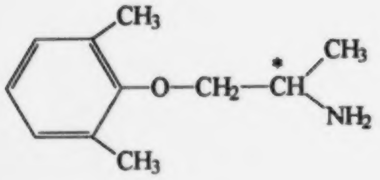
Figure 3- 10. Metabolism of *rac*-preprenalterol, by *C. echinulata* 4145, at 1% of atmospheric oxygen levels.

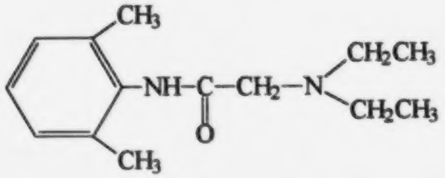
4.0 Fungal Biotransformation of *rac*-Mexiletine

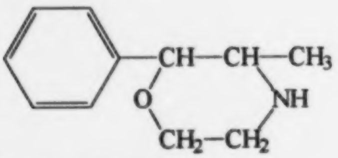
4.1 Introduction

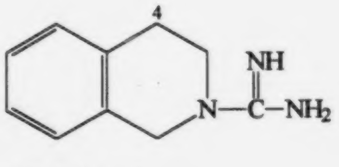
rac-Mexiletine (Figure 4-1A) is a class 1b antiarrhythmic drug, similar in structure to lidocaine (Figure 4-1B), used in the treatment of ventricular arrhythmias. Mexiletine's half-life ranges from 6 to 12 hours compared to a range of 1.2 to 1.9 hours for lidocaine, making it an orally effective agent (Schraeder and Bauman 1986). *rac*-Mexiletine was the product of a long development process started in the early 1960's. It began when chemists, at Boehringer Ingelheim, were asked to synthesize a new anorectic compound with decreased central nervous system (CNS) activity. The structure of phenmetrazine was to be used as a template for this new drug (Figure 4-1C, Shanks 1977). Unfortunately, their research demonstrated that modifications to phenmetrazine's structure removed all anorectic activity. However, additional testing revealed that the modified compounds had anticonvulsant activity due to di-substitutions of the phenyl ring. This effort culminated in a patent application for an anticonvulsive (anti-epileptic) possessing minimal CNS effects (called Ko1173 or *rac*-mexiletine, Koppe 1977). Additional testing revealed that this compound exhibited antiarrhythmic activity leading to its use as a prophylactic treatment for post-infarction patients who are likely to develop ventricular arrhythmias. This is a good example of a serendipitous discovery in science.

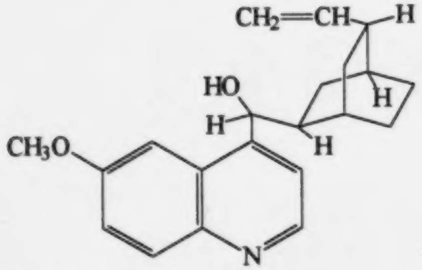
Antiarrhythmic drugs are classified according to their predominant effect on the action potential of Purkinje fiber cells in the heart (Harvey *et al.* 1992). Class 1b drugs are grouped together because of their direct action on cellular membranes. They function by decreasing the rate of sodium entry into the cell during the repolarization phase of an action potential (Williams 1975). This causes a decrease in cell excitability and a slowing of conduction velocity. Therefore, class 1b agents, including *rac*-mexiletine, are useful for the treatment of cardiac arrhythmias. Some favorable characteristics of *rac*-mexiletine therapy include a lack of effect on the autonomic nervous system and acetylcholine activity in addition to its inability to block α or β receptors. However, adverse effects include gastrointestinal distress, lightheadedness, and tremors (Campbell *et al.* 1977).


A] rac-Mexiletine
* Denotes chiral center


B] Lidocaine


C] Phenmetrazine


D] Debrisoquine


E] Quinidine

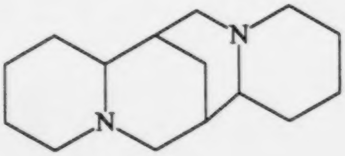

F] Sparteine

Figure 4-1. Chemical structures of the drugs *rac*-mexiletine, lidocaine, phenmetrazine, debrisoquine, *rac*-quinidine, and sparteine.

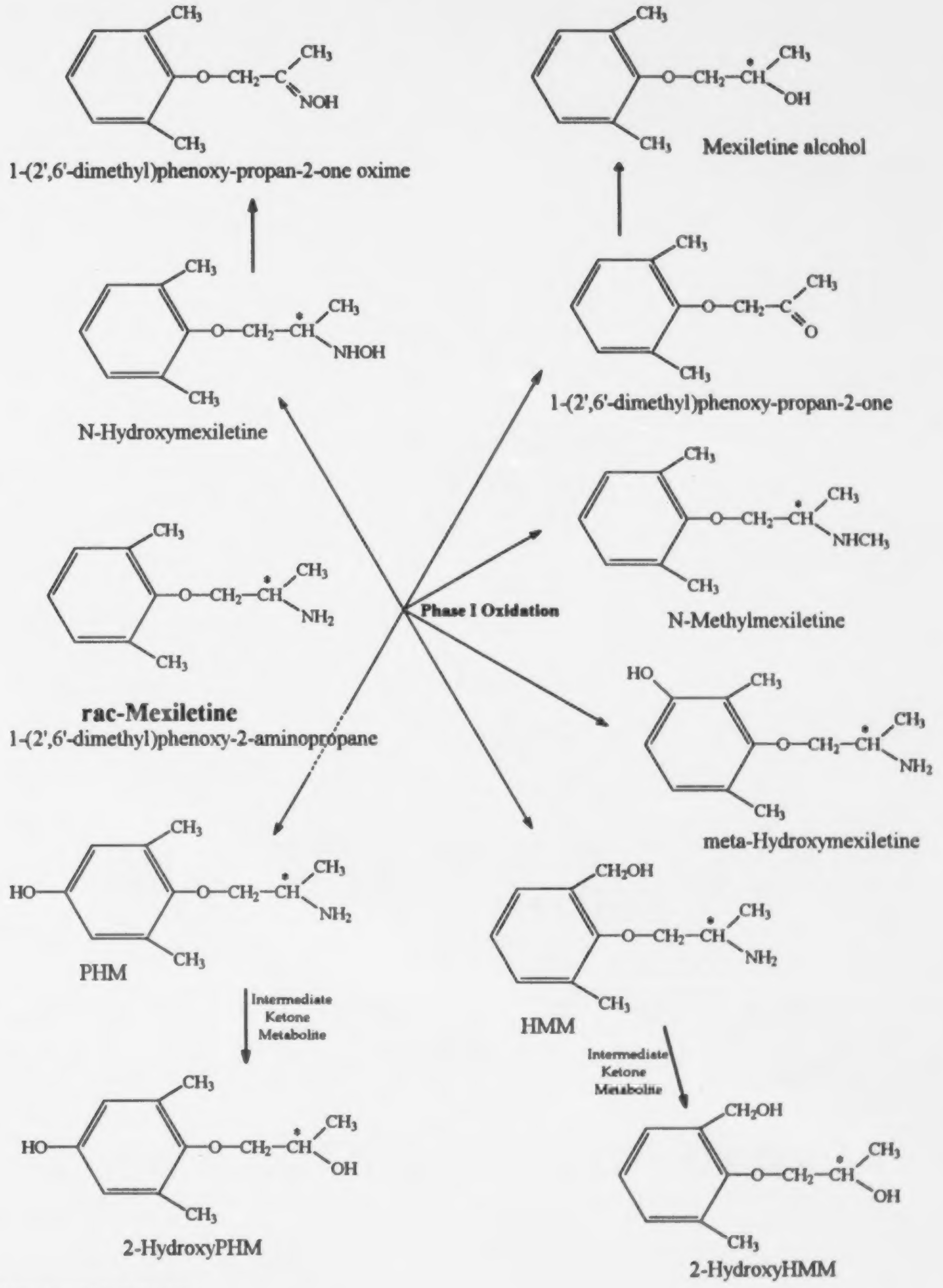
rac-Mexiletine is an orally administered, weakly basic (pKa 8.75) drug, with a narrow therapeutic range, where serum concentrations must remain between 0.8 and 2.0 mg/L (Campbell *et al.* 1978). In healthy volunteers, it is almost completely absorbed (bioavailability is 88%) with peak plasma concentrations occurring within 2 to 4 hours (Haselbarth *et al.* 1981). In coronary artery disease patients, these numbers are lower due to decreased gastric emptying (Kelly 1977). In addition, mexiletine is administered as a racemate though no significant difference between the enantiomers has been reported for the absorption rate constant, peak plasma concentration, nor the time required to achieve peak plasma concentrations (Igwemezie *et al.* 1989). However, it has been reported there are differences in serum protein binding (McErlane *et al.* 1987), receptor binding (Hill *et al.* 1988), electrophysiology (Turgeon *et al.* 1991), excretion (Grech-Belanger *et al.* 1986) and metabolism (Vandamme *et al.* 1993).

An interesting feature of *rac*-mexiletine therapy is the large interindividual variations occurring in plasma concentrations with all modes of administration (Gillis and Kates 1984). One reported source of variation was urine pH. Mitchell *et al.* (1983) demonstrated that decreasing urine pH from 8.0 to 5.0 produced a consistent increase in renal clearance. This correlated with mean plasma concentrations which were measured as 1.63 μg/mL for acidic urine and 1.30 μg/mL for alkaline urine. This difference occurred because, as urine acidity decreases, there is greater renal absorption of the unionized drug (Beckett and Chidomere 1977a). Therefore, in order to assess pharmacokinetic parameters between patients, urine pH must be standardized. Other sources of variation included patient age (Grech-Belanger *et al.* 1989), smoking (Grech-Belanger *et al.* 1985), and use of additional medications including cimetidine (Klein *et al.* 1988) and rifampicin (Pentikainen *et al.* 1982).

The predominant factor influencing plasma concentrations of *rac*-mexiletine was the liver's capacity to process the drug. Less than 10% of an oral dose was excreted unchanged in urine over three days, emphasizing the importance of metabolism (Prescott *et al.* 1977). The body generates numerous metabolites through various pathways including oxidation, reduction, deamination, and conjugation. The principal metabolites (20% of an administered dose) are formed via oxidation of *rac*-mexiletine to PHM, HMM, and their corresponding alcohols N-hydroxy-PHM (N-PHM) and N-hydroxy-HMM (N-HMM, Beckett and Chidomere 1977b). However, total urinary recovery of the drug, PHM, HMM, N-PHM, N-HMM, and their conjugated phase II metabolites only accounts for 30% of an administered dose. Turgeon *et al.* (1992), recovered an additional 40% as two glucuronide metabolites formed after N-oxidation and deamination of *rac*-mexiletine. A summary of *rac*-mexiletine's metabolic profile in humans is outlined in figures 4-2 and 4-3.

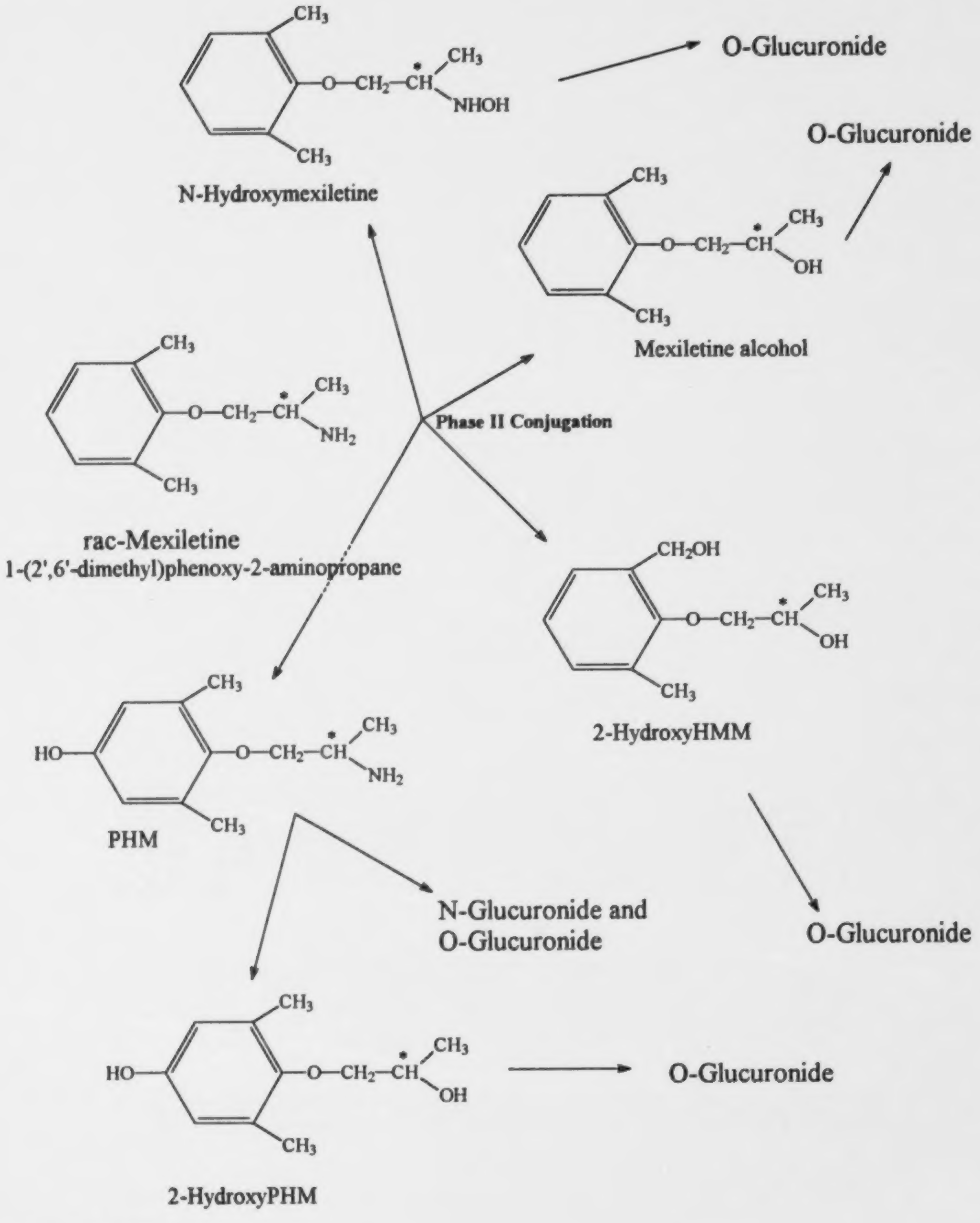
Although the pathways involved in *rac*-mexiletine's metabolism were elucidated by 1988, the enzymes concerned were not. Therefore, Broly *et al.* (1989) investigated *rac*-mexiletine's metabolism *in vitro* using human liver microsomes. This study established that liver preparations were capable of oxidizing *rac*-mexiletine to HMM and PHM. Further testing narrowed their biosynthesis to a cytochrome P_{450} enzyme because their production could be inhibited by heating the reaction mixture, adding carbon monoxide, and not adding NADPH. In addition, specific cytochrome P_{450} inhibitors, including SKF525-A, metyrapone, α-naphthoflavone, and quinidine (Figure 4-1E), were used to determine that the isoenzyme involved was cytochrome P450 db1 (P_{450} IID1, P_{450} bufI). This particular enzyme is also responsible for debrisoquine (Figure 4-1D)/sparteine (Figure 4-1F) type polymorphism and is now known as CYP2D6 (Brosen 1990).

Metabolic polymorphism was a concept used to explain the discovery of two distinct populations differing in their ability to metabolize debrisoquine and sparteine (Mahgoub *et al.* 1977, Eichelbaum *et al.* 1979). If a study was conducted on a large number of people given the drugs debrisoquine or sparteine, two distinct groups would be observed. The first, called extensive metabolizers (EMs), excrete 10 to 200 times more of the metabolites 4-hydroxydebrisoquine or sparteine N-oxide than the second group known as poor metabolizers (PMs). About 10% of Caucasians belong to the PM phenotype (Steiner *et al.* 1985). Upon


1-(2',6'-dimethyl)phenoxy-propan-2-one oxime
Mexiletine alcohol
N-Hydroxymexiletine
1-(2',6'-dimethyl)phenoxy-propan-2-one
N-Methylmexiletine
Phase I Oxidation
rac-Mexiletine
1-(2',6'-dimethyl)phenoxy-2-aminopropane
meta-Hydroxymexiletine
PHM
HMM
Intermediate Ketone Metabolite
Intermediate Ketone Metabolite
2-HydroxyPHM
2-HydroxyHMM
CH3
NOH
OH
NHOH
O
NH2
NHCH3
HO
CH2OH

* Denotes Chiral Center

Figure 4-2. A summary of the phase I oxidative metabolites detected in human plasma and urine after administration of *rac*-mexiletine.



N-Hydroxymexiletine
O-Glucuronide
O-Glucuronide
Mexiletine alcohol
rac-Mexiletine
1-(2',6'-dimethyl)phenoxy-2-aminopropane
Phase II Conjugation
2-HydroxyHMM
PHM
N-Glucuronide and
O-Glucuronide
O-Glucuronide
2-HydroxyPHM
O-Glucuronide

* Denotes Chiral Center

Figure 4-3. A summary of the phase II conjugated metabolites, along with their phase I precursors, detected in human plasma and urine after administration of *rac*-mexiletine.

closer examination, family studies established that the deficiency occurring in PMs was under genetic control (Evans *et al.* 1980). In addition, because the reaction was oxidative, it was suggested that a defective cytochrome P_{450} could be present in the PM. In 1988, Gonzalez *et al.* elucidated the exact mechanism of polymorphism by demonstrating that PMs of debrisoquine have negligible amounts of CYP2D6. Furthermore, they identified three defective mRNA's as the cause of the aberrant enzyme activity. This discovery demonstrated the mechanism by which PMs exhibit an impaired capacity for the oxidative metabolism of debrisoquine and sparteine. In addition, at least 20 other drugs, including β-blockers and antiarrhythmics, have been shown to be substrates for this isoenzyme. The clinical significance of this finding is that PMs could be at risk from side effects associated with increased plasma concentrations of these drugs.

The role of debrisoquine/sparteine polymorphism in *rac*-mexiletine metabolism was examined in more detail by Turgeon *et al.* (1991). Using 14 healthy volunteers (10 EMs and 4 PMs) they wanted to determine whether low doses of quinidine, a CYP2D6 inhibitor, could repress this enzyme and turn EMs into PMs after oral administration of *rac*-mexiletine. When the drug was administered alone, mean plasma concentrations and urinary recovery of HMM and PHM were higher in PMs compared to EMs. However, there was no difference in formation of N-hydroxy metabolites. With co-administration of quinidine, the kinetics of *rac*-mexiletine were unchanged in PMs. In EMs, the kinetics were altered to the point where no difference was observed between EMs and PMs. On average, quinidine co-administration impaired oxidative carbon metabolism by 70% but had no effect on nitrogen oxidation. Therefore, this provided evidence for the role of CYP2D6 in *rac*-mexiletine metabolism *in vivo.*

An aspect of *rac*-mexiletine administration not yet discussed in detail is whether the enantiomers have different properties *in vivo.* This is an important issue which has been examined in detail with other chiral drugs (Jamali *et al.* 1991). Studies that have characterized enantiomeric differences for *rac*-mexiletine are summarized below. The preferred enantiomer, for actions listed in the left hand column, are indicated with a √ :

	R-(-)-Mexiletine	S-(+)-Mexiletine
Serum protein binding (Igwemezie *et al.* 1987).	√	
Cardiac sodium channel binding (Hill *et al.* 1988).	√	
Cardiac uptake in rat (Igwemezie *et al.* 1991).	No	Difference
Antiarrhythmic activity (Turgeon *et al.* 1991).	√	
Glucuronide excretion (Grech-Belanger *et al.* 1986).	√	

In addition to these findings, human liver microsomes were used to investigate stereoselective aspects of *rac*-mexiletine's metabolism *in vitro* (Vandamme *et al.* 1993). It was demonstrated that p-hydroxylation to PHM was the favored product from S-(+)-mexiletine and aliphatic hydroxylation to HMM was preferred with R-(-)-mexiletine. It was also observed that biosynthesis of these metabolites was inhibited by quinidine and that dextromethorphan O-demethylation was competitively inhibited by S-(+)- and R-(-)-mexiletine. This provided supplementary evidence for the role of CYP2D6 in stereoselective aspects of *rac*-mexiletine's oxidative metabolism.

4.1.1 Objectives

These studies have provided detailed information regarding the pathways of *rac*-mexiletine metabolism in humans and the specific enzymes involved. As was discussed in the introduction, fungal models of mammalian metabolism are useful tools to study drug metabolism *in vitro*. This ability exists because fungi possess a cytochrome P_{450} enzyme system analogous to the one found in human livers. Therefore, they can be used to generate a metabolic profile of the possible oxidative metabolites prior to human administration. We wanted to test this model by determining whether the fungus C. *echinulata* 4145 was capable of metabolizing *rac*-mexiletine in a fashion similar to *in vivo* and *in vitro* results discussed. In addition, a stereospecific assay has been developed (Freitag *et al.* 1993) to simultaneously resolve the enantiomers of mexiletine, HMM, and PHM. Therefore, it was possible to determine whether metabolism was stereoselective. Finally, since we have stated that an important advantage of fungal models was the ability to scale up a desired reaction, we wanted to optimize conditions corresponding to maximum metabolite production.

4.2 Results

As was discussed in chapter 3, fungal growth can be influenced by environmental conditions. When employing submerged culture techniques, growth can range from individual pellets to filamentous mycelia dispersing evenly throughout a medium. The exact morphology will be influenced by physical parameters including agitation and the components chosen for the growth medium. Consequently, if these factors can influence visible aspects of fungal growth, they should also be affecting the cell's biochemistry. Therefore, experiments were designed to test the influence of medium components on biotransformation reactions with the goal of finding optimal conditions for metabolite formation.

4.2.1 Effect of Nitrogen Source

In preliminary work, we found that the nitrogen source employed influenced fungal growth considerably. We also established that C. *echinulata* 4145 was capable of biotransforming *rac*-mexiletine into HMM and PHM. Therefore, we combined these two experiments and investigated the effect of different nitrogen sources, at pH 7.0, on the conversion of *rac*-mexiletine into HMM and PHM. The nitrogen sources tested were as follows:

	Nitrogen Source
Yeast Extract (pH 7.0).	Water soluble proteins remaining after yeast cell autolysis (10% nitrogen).
Trypticase Soy (pH 7.0).	Pancreatic digest of casein. Papaic digest of soy meal.
Phase I: trypticase Soy (pH 7.0). Phase II: phosphate Buffer (pH 7.0).	No nitrogen source in phase II.
Casamino Acids (pH 7.0).	Acid hydrolysed casein (10% nitrogen).
Czapec Dox (pH 7.0).	Sodium nitrate (0.3% nitrogen).
Malt Extract (pH 7.0).	Water soluble compounds from malt.
Peptone (pH 7.0).	Water soluble proteins remaining after digestion of animal tissue (16% nitrogen) with enzymes (pepsin).
Sabouraud dextrose (pH 7.0).	Neopeptone which contains: Pancreatic digest of casein. Peptic digest of animal tissue.

This experiment was performed in triplicate for each nitrogen source listed to insure the validity of our results. Biotransformations were achieved in 125 mL Erlenmeyer flasks, containing 25 mL of media, grown at 28 °C in an environmental shaker. The organism used for all experiments was *Cunninghamella echinulata* 4145. The amount of drug added to each flask was identical. A stock solution of *rac*-mexiletine was prepared, filter sterilized (22 μm pore size), and the exact drug content determined using HPLC. We found that this determination was necessary because the drug was adsorbing onto the filter matrix. The concentration of *rac*-mexiletine added to each flask was determined to be 197.6 μg/mL. The averages of the results obtained from these experiments are as follows (Tables 4-1 to 4-16, Figures 4-4 to 4-19):

Table 4-1. Metabolism of *rac*-mexiletine in yeast extract broth at pH 7.0.

Average concentration

	Drug Enantiomer Concentration (μg/mL)					
Time	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	69.8	69.2	0.0	0.0	0.0	0.0
12	61.4	56.9	5.5	5.6	8.0	11.9
21	54.8	44.2	6.1	6.8	8.0	17.1
29	39.4	29.4	8.7	8.1	10.3	21.0
36	36.9	25.0	11.3	9.8	11.6	28.1
45	34.8	20.9	13.6	11.1	14.4	32.9
53	31.3	17.0	14.9	11.6	16.0	36.0
60	25.9	13.6	14.3	11.0	15.8	33.5
69	22.4	11.4	14.5	10.8	18.9	39.2
76	22.7	10.1	15.4	10.8	17.5	35.8
83	23.2	10.3	17.8	12.3	19.9	40.5
93	18.9	7.9	16.6	12.4	18.9	33.8
105	14.9	7.6	14.9	9.9	22.5	37.7
118	14.7	6.8	17.8	11.2	24.1	42.5
129	12.7	6.5	17.7	12.6	21.4	38.9
142	11.4	6.3	17.6	10.3	22.1	39.2
166	11.6	6.2	21.2	11.8	26.9	48.1
200	9.3	5.9	17.4	9.9	23.0	39.6

Table 4-2. A summary of data obtained from the metabolism of *rac*-mexiletine in yeast extract broth.

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	14.9	105	15.2%
R-(-)-Mexiletine	7.6	105	7.7%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	17.8	83	18.1%
R-HMM	12.3	83	12.5%
S-PHM	24.1	118	24.5%
R-PHM	39.2	69	39.9%
Total recovery of added drug (Total amount recovered/amount added x 100			59.4%

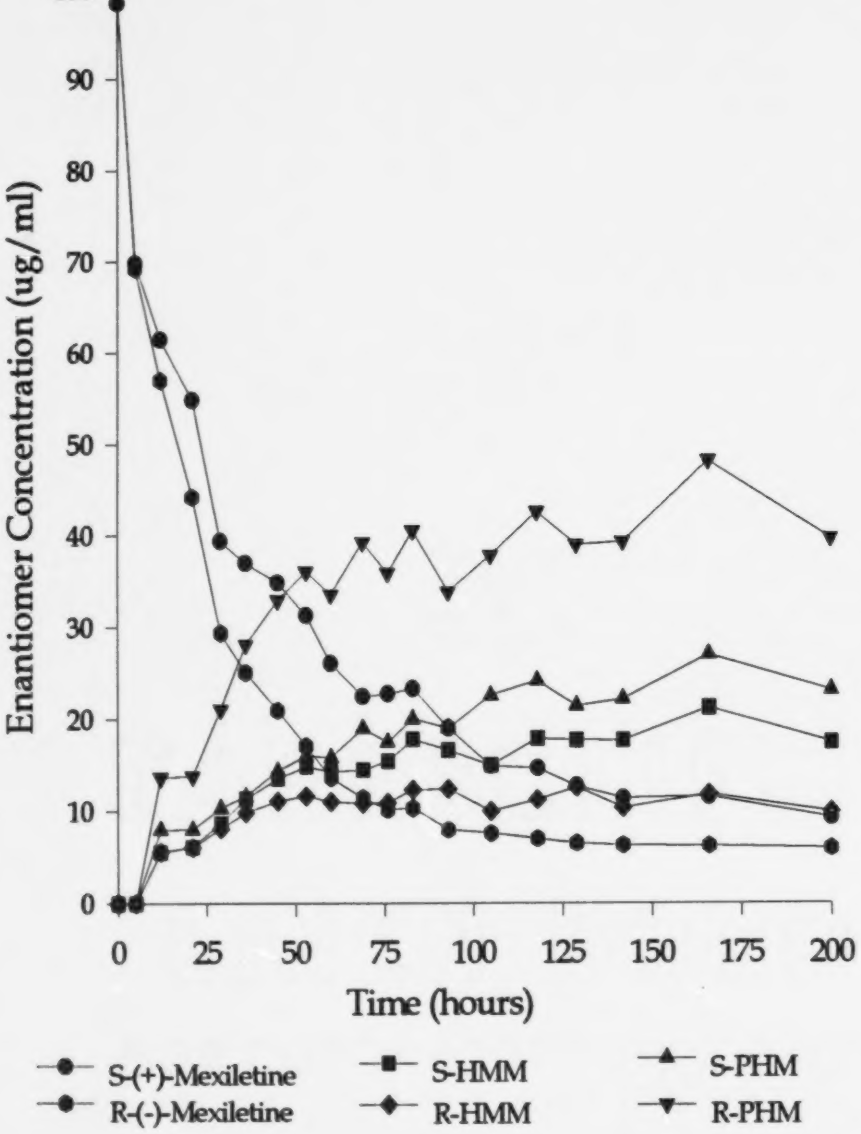


Figure 4-4. A concentration time profile of *rac*-mexiletine, *rac*-HMM, and *rac*-PHM enantiomers in yeast extract broth.

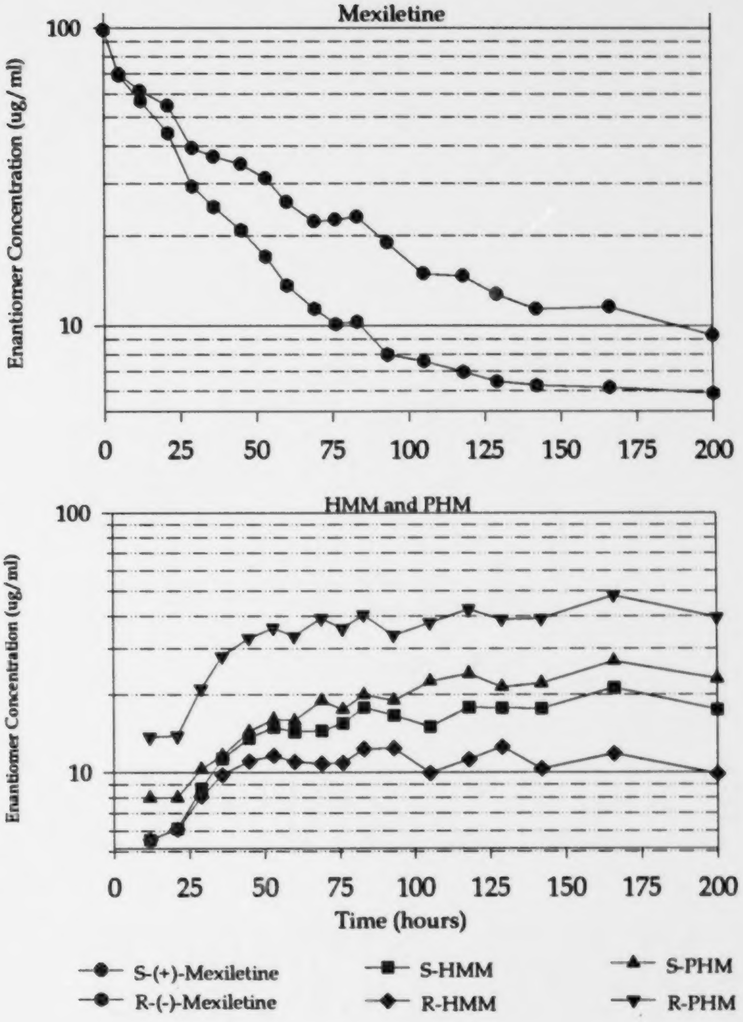


Figure 4-5. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in **yeast extract broth.**

Table 4- 3. Metabolism of *rac*-mexiletine in **trypticase soy broth** at pH 7.0.

Average concentration

	Drug Enantiomer Concentration (μg/mL)					
Time	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	45.9	45.9	0.0	0.0	0.0	0.0
12	30.6	24.6	10.3	9.3	10.9	20.8
21	21.1	14.7	12.7	11.6	15.3	32.2
29	14.7	11.2	11.3	8.9	14.9	30.1
36	13.1	10.4	10.8	8.7	14.5	28.4
45	12.4	10.3	10.3	8.3	14.6	28.1
53	12.1	10.2	9.9	8.2	14.5	27.6
60	11.4	10.1	10.2	8.2	15.7	28.7
69	11.8	10.2	9.9	8.3	15.3	28.8
76	11.7	10.1	10.3	8.2	15.8	29.7
83	11.8	10.2	10.0	8.2	15.2	28.7
93	11.8	9.9	10.1	8.1	15.2	28.8
105	11.8	9.9	10.2	7.9	15.4	29.1
118	12.4	9.9	11.1	8.4	17.3	34.6
129	11.9	10.2	10.7	8.9	16.5	32.3
142	11.6	9.9	10.0	8.0	14.7	28.3
166	12.4	10.4	10.7	8.3	16.8	32.8
200	11.4	10.1	9.9	7.9	14.9	27.1

Table 4-4. A summary of data obtained from the metabolism of *rac*-mexiletine in trypticase soy broth.

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	13.4	36	13.6%
R-(-)-Mexiletine	10.4	36	10.6%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	12.7	21	12.9%
R-HMM	11.6	21	11.8%
S-PHM	15.3	21	15.6%
R-PHM	32.2	21	32.8%
Total recovery of added drug (Total amount recovered/amount added x 100			49.5%

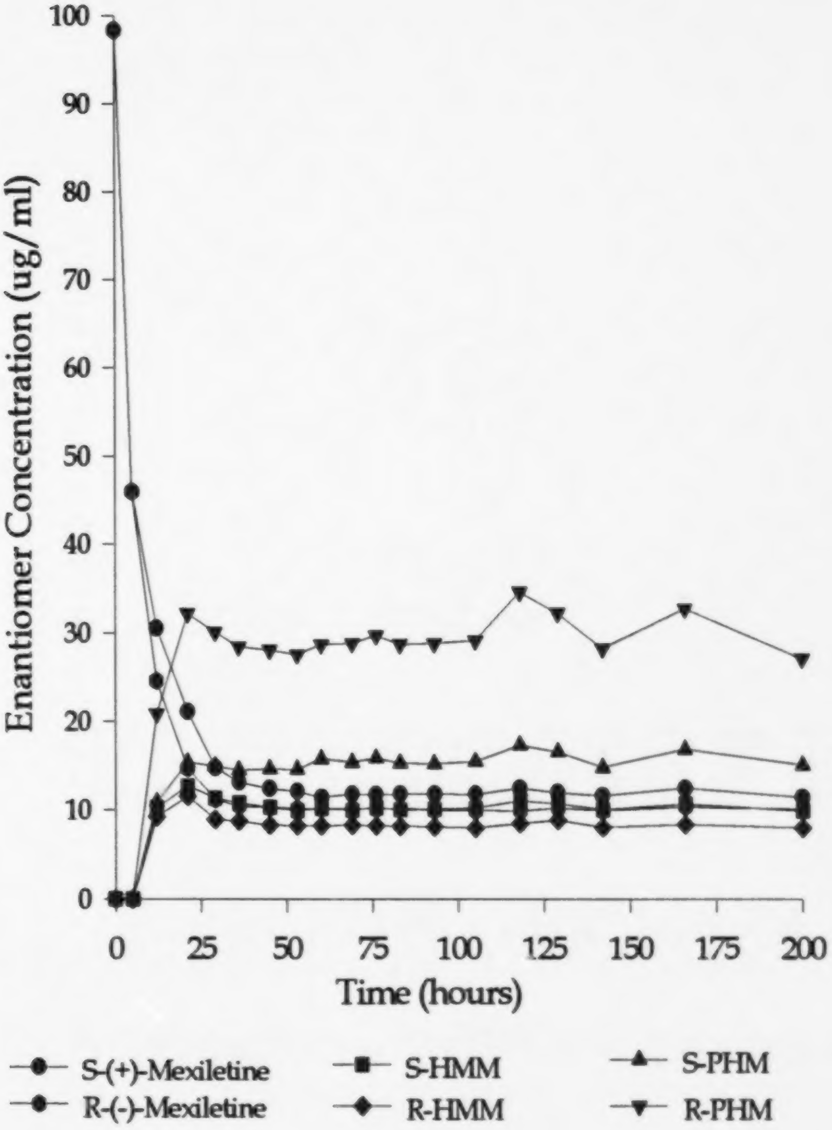


Figure 4-6. A concentration time profile of mexiletine, HMM, and PHM enantiomers in trypticase soy broth.

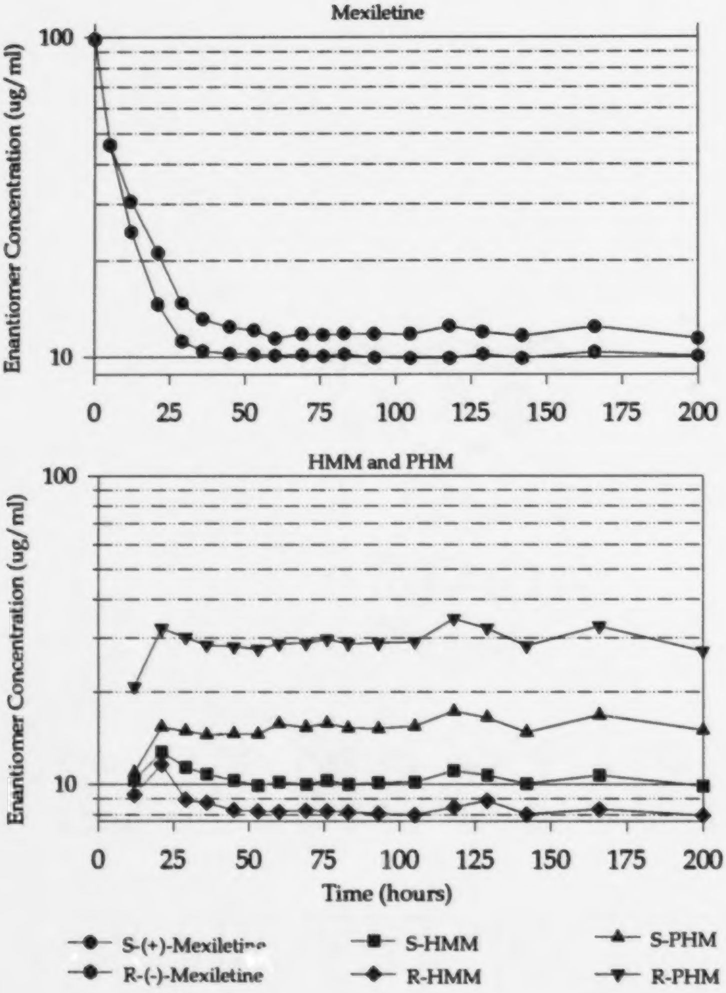


Figure 4-7. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in **trypticase soy broth.**

Table 4-5. Metabolism of *rac*-mexiletine using trypticase soy broth for phase I and **phosphate buffer** (pH 7.0) for phase II.

Average concentration

Time	Drug Enantiomer Concentration (μg/mL)					
	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	55.5	55.3	0.0	0.0	0.0	0.0
12	49.2	47.8	6.3	5.9	6.8	9.7
21	39.2	36.8	6.8	6.6	7.8	12.0
29	30.2	27.7	6.8	6.7	8.1	13.1
36	33.9	28.9	7.4	7.3	8.6	15.9
45	32.2	28.7	8.1	7.7	9.3	17.1
53	26.5	23.6	7.6	7.3	9.1	16.0
60	27.2	24.1	7.9	7.6	9.4	18.4
69	29.7	25.9	8.0	7.8	9.4	19.1
76	28.8	25.2	8.5	8.1	10.2	20.4
83	25.9	22.8	7.7	7.4	9.1	18.2
93	22.9	20.2	7.4	7.1	9.1	17.4
105	24.9	21.8	8.1	7.6	9.7	19.6
118	28.5	24.7	9.0	8.3	11.0	23.9
129	31.6	27.4	9.6	8.9	11.7	26.4
142	22.9	20.3	7.9	7.7	10.9	23.8
166	27.3	22.7	9.3	8.8	12.9	26.6
200	25.6	21.3	8.5	8.2	12.1	24.1

Table 4- 6. A summary of data obtained from the metabolism of *rac*-mexiletine in **phosphate buffer**.

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	26.5	53	26.9%
R-(-)-Mexiletine	23.6	53	24.0%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	8.1	45	8.2%
R-HMM	7.7	45	7.8%
S-PHM	10.2	76	10.4%
R-PHM	20.4	76	20.8%
Total recovery of added drug (Total amount recovered/amount added x 100			49.1%

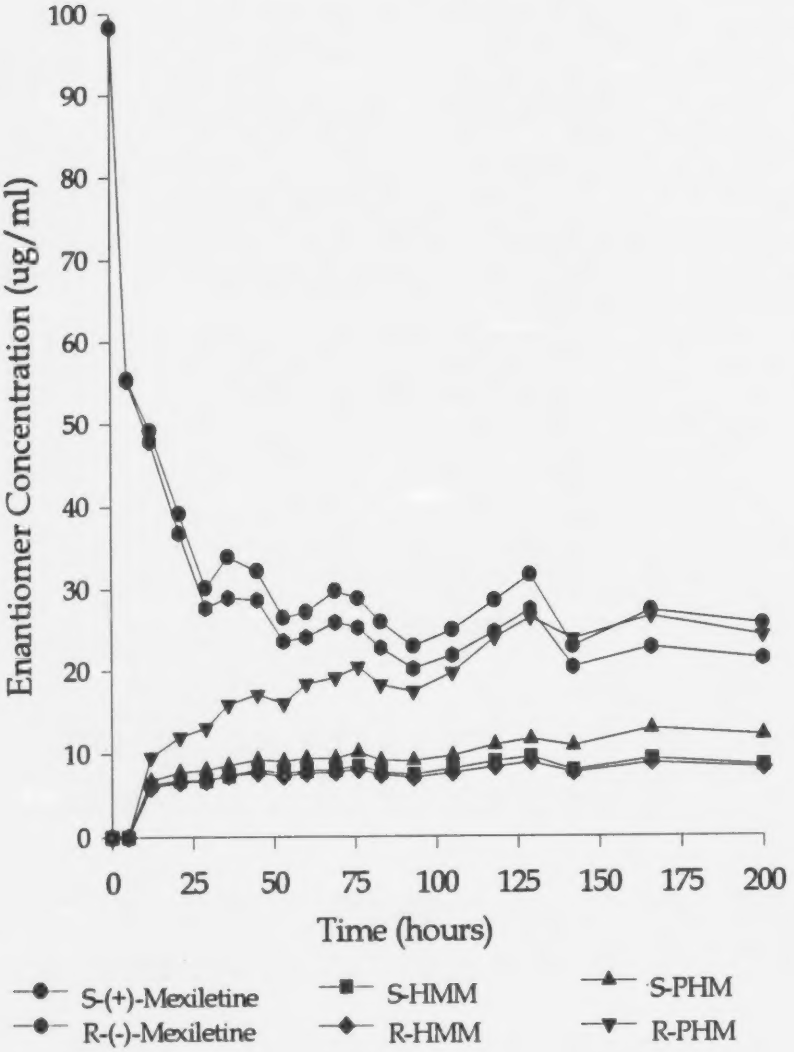


Figure 4-8. A concentration time profile of mexiletine, HMM, and PHM enantiomers in **phosphate buffer** (pH 7.0).

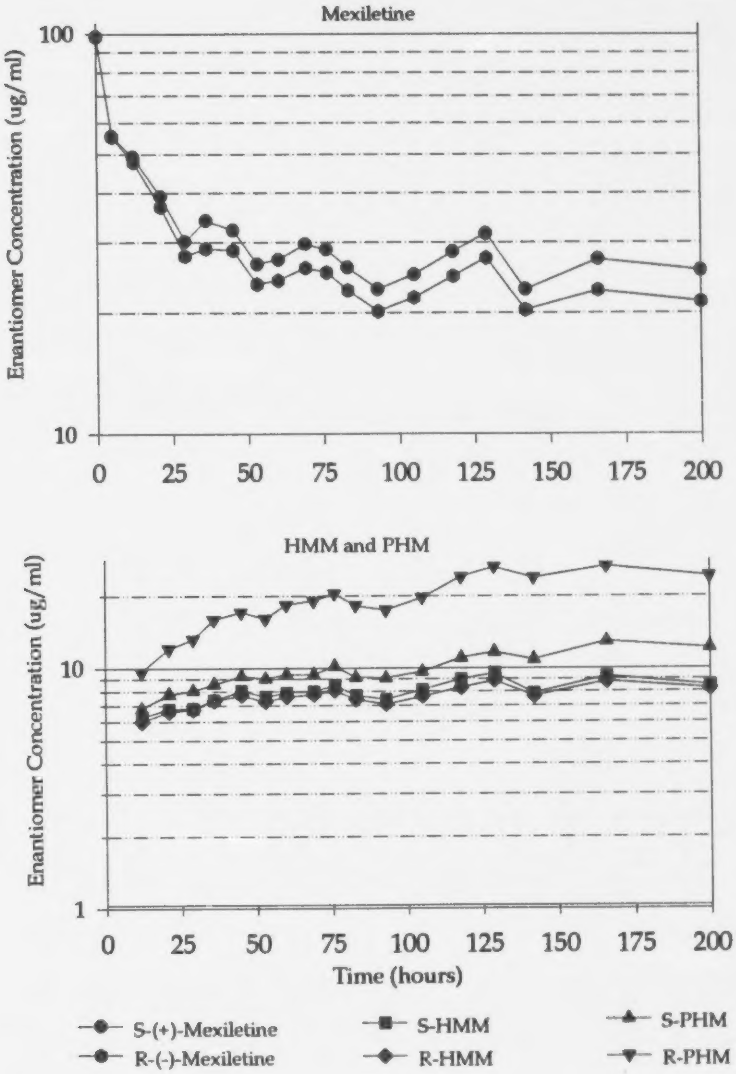


Figure 4-9. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in **phosphate buffer** (pH 7.0).

Table 4-7. Metabolism of *rac*-mexiletine in **casamino acid broth** at pH 7.0.

Average concentration

	Drug Enantiomer Concentration (µg/mL)					
Time	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	74.6	74.9	0.0	0.0	0.0	0.0
12	83.5	82.9	0.0	0.0	0.0	0.0
21	69.6	68.6	0.0	0.0	0.0	0.0
29	80.1	79.1	0.0	0.0	0.0	6.2
36	74.4	73.6	1.9	0.0	0.0	7.3
45	76.3	74.3	2.6	2.9	5.6	7.6
53	82.0	79.5	3.2	2.7	6.3	9.5
60	78.5	74.6	3.2	3.4	6.1	9.6
69	74.4	69.2	3.02	3.5	6.0	9.7
76	81.4	77.0	3.6	3.7	6.8	10.7
83	69.5	61.3	3.2	3.6	6.5	9.9
93	70.8	65.2	3.8	3.9	7.1	11.9
105	71.9	64.1	4.5	4.4	7.6	14.1
118	66.4	58.3	4.9	4.8	7.5	15.9
129	73.5	60.4	6.3	5.8	9.3	20.1
142	63.9	54.9	6.7	6.3	10.1	22.5
166	46.9	37.9	8.3	7.2	12.1	27.9
200	44.5	29.8	9.5	7.7	14.3	34.0

Table 4-8. A summary of data obtained from the metabolism of *rac*-mexiletine in **casamino acid broth.**

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	44.6	200	45.3%
R-(-)-Mexiletine	29.8	200	30.2%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	9.5	200	9.6%
R-HMM	7.7	200	7.8%
S-PHM	14.3	200	14.5%
R-PHM	34.0	200	34.6%
Total recovery of added drug (Total amount recovered/amount added x 100			71.0%

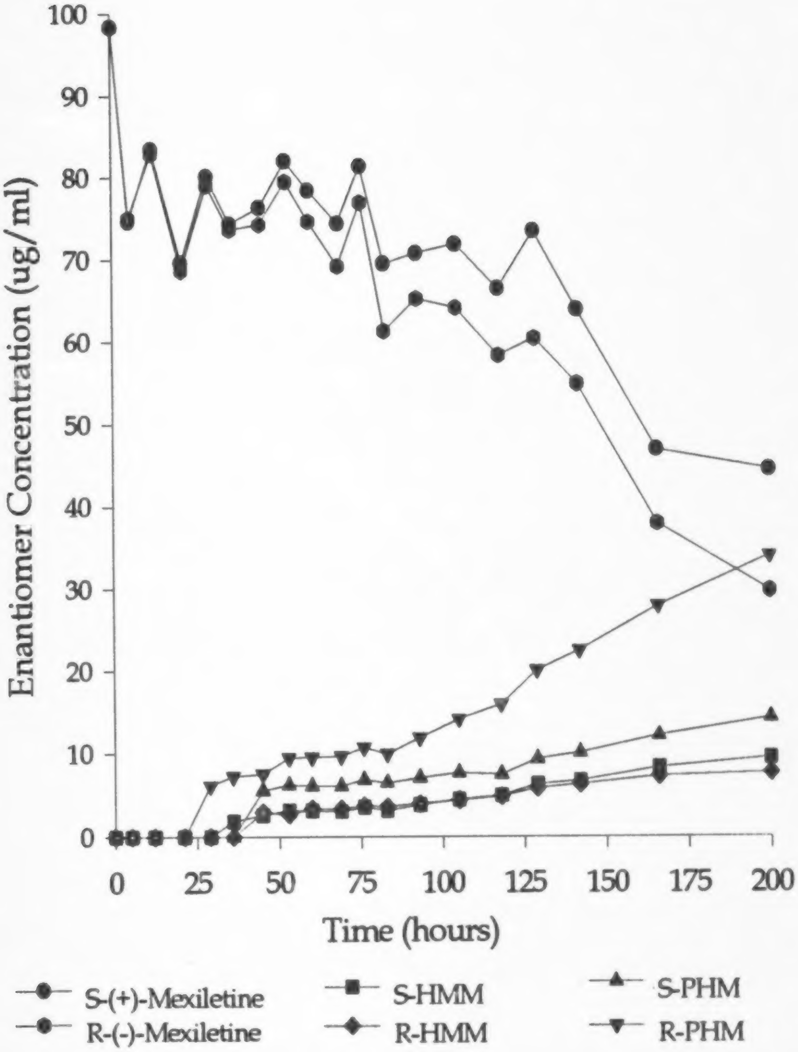


Figure 4-10. A concentration time profile of mexiletine, HMM, and PHM enantiomers in **casamino acid broth** (pH 7.0).

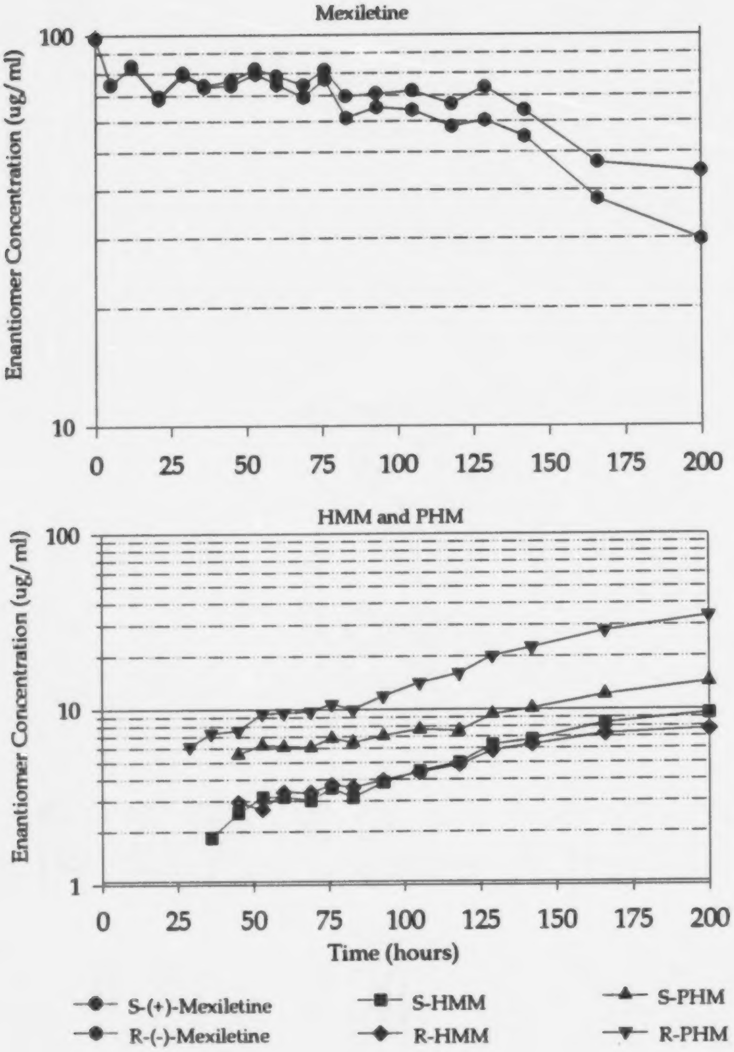


Figure 4-11. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in **casamino acid broth** (pH 7.0).

Table 4-9. Metabolism of *rac*-mexiletine in **czapec dox broth** at pH 7.0.

Average concentration

	Drug Enantiomer Concentration (μg/mL)					
Time	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	80.0	80.8	0.0	0.0	0.0	0.0
12	90.7	91.2	6.9	5.1	0.0	9.9
21	77.7	75.5	6.9	6.9	7.8	11.9
29	68.5	65.2	7.0	7.1	8.5	13.2
36	63.2	58.5	7.7	7.8	9.3	14.9
45	62.7	56.8	8.3	8.3	9.8	16.9
53	59.8	50.3	9.1	9.0	10.5	19.9
60	53.5	47.3	9.2	8.9	10.3	20.3
69	56.3	49.2	9.3	9.3	10.7	21.1
76	64.1	55.5	10.0	9.5	11.4	22.8
83	45.9	39.7	9.1	8.8	10.0	19.26
93	55.7	47.6	10.0	9.6	11.1	22.5
105	43.6	36.2	9.9	9.4	11.5	22.4
118	48.6	40.1	10.9	10.3	11.8	25.6
129	43.6	35.8	10.8	10.5	12.1	25.6
142	45.0	37.8	11.4	10.6	12.3	27.2
166	41.3	32.2	12.2	11.3	13.5	29.6
200	39.2	29.9	12.8	11.7	14.2	28.9

Table 4- 10 A summary of data obtained from the metabolism of *rac*-mexiletine in **czapec dox broth.**

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	43.6	105	44.3%
R-(-)-Mexiletine	36.2	105	36.8%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	10.9	118	11.1%
R-HMM	10.3	118	10.5%
S-PHM	11.9	118	12.1%
R-PHM	25.7	118	26.1%
Total recovery of added drug (Total amount recovered/amount added x 100			70.4%

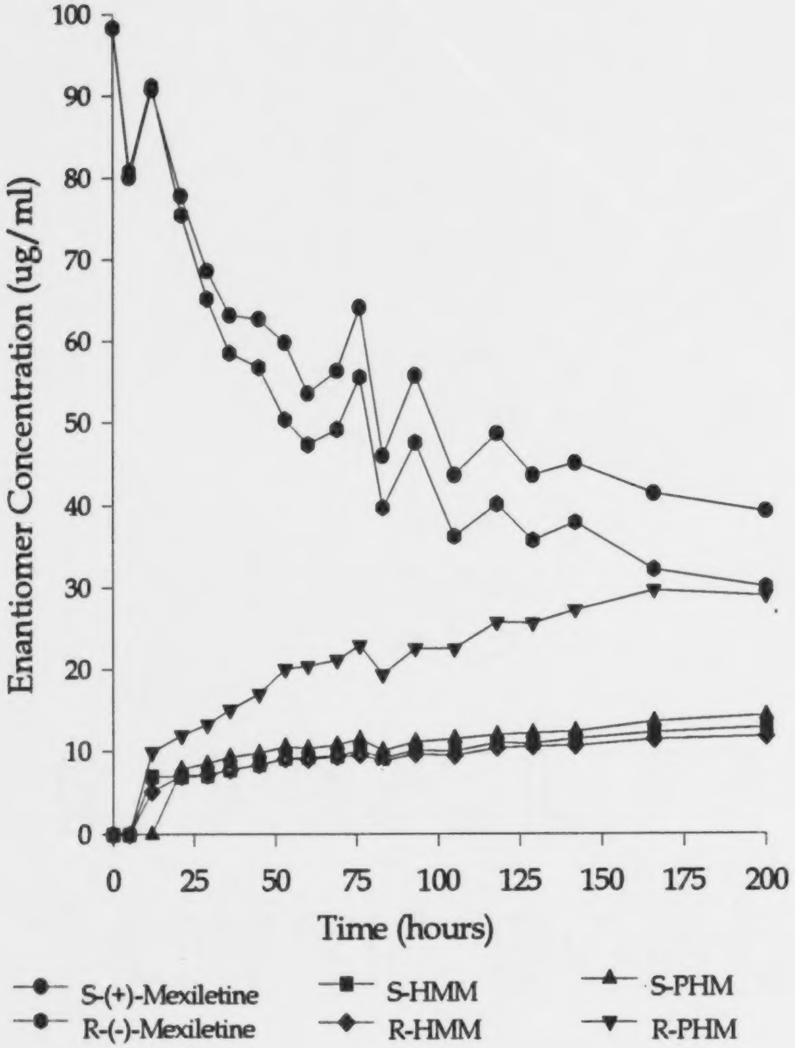


Figure 4-12. A concentration time profile of mexiletine, HMM, and PHM enantiomers in czapec dox broth (pH 7.0).

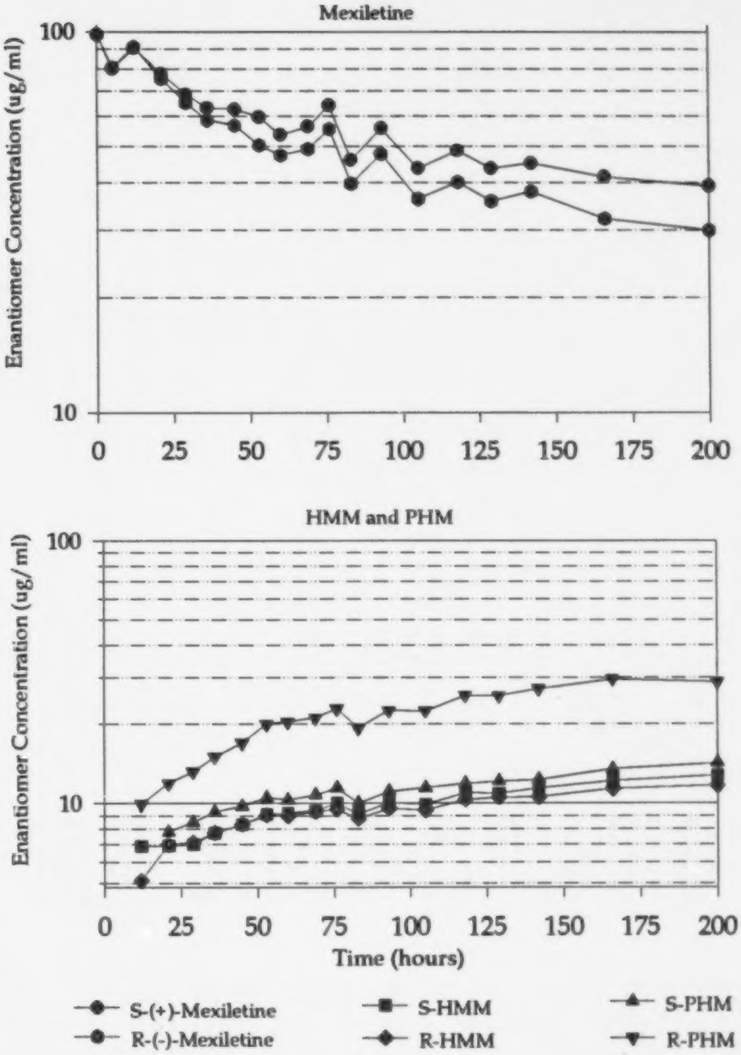


Figure 4-13. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in czapec dox broth (pH 7.0).

Table 4-11. Metabolism of *rac*-mexiletine in **malt extract broth** at pH 7.0.

Average concentration

	Drug Enantiomer Concentration (µg/mL)					
Time	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	61.7	62.7	0.0	0.0	0.0	0.0
12	69.7	71.0	0.0	0.0	0.0	0.0
21	62.2	64.4	0.0	0.0	0.0	0.0
29	56.1	56.0	2.8	3.4	2.4	2.0
36	63.2	63.1	3.3	3.9	4.0	5.4
45	65.8	65.4	2.4	2.7	3.6	7.5
53	64.9	64.3	3.4	4.1	4.1	7.9
60	63.1	62.2	3.7	4.3	4.4	7.2
69	63.4	61.6	3.7	4.2	4.4	7.1
76	74.1	72.2	2.6	3.2	4.2	6.1
83	45.1	43.6	5.7	4.2	4.4	7.0
93	57.3	54.7	5.7	4.7	5.0	9.1
105	50.4	47.5	6.0	4.3	4.6	8.0
118	47.6	45.1	5.7	6.4	6.2	11.2
129	60.3	57.0	4.6	4.9	5.3	10.2
142	56.6	52.5	4.5	4.9	5.3	10.3
166	53.0	49.0	4.8	5.1	5.5	10.8
200	56.2	50.8	5.4	5.7	6.1	13.2

Table 4-12. A summary of data obtained from the metabolism of *rac*-mexiletine in **malt extract broth.**

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	45.2	21	45.9%
R-(-)-Mexiletine	45.9	21	46.7%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	4.9	105	4.9%
R-HMM	5.2	105	5.3%
S-PHM	6.9	105	7.0%
R-PHM	10.5	105	10.7%
Total recovery of added drug (Total amount recovered/amount added x 100			60.3%

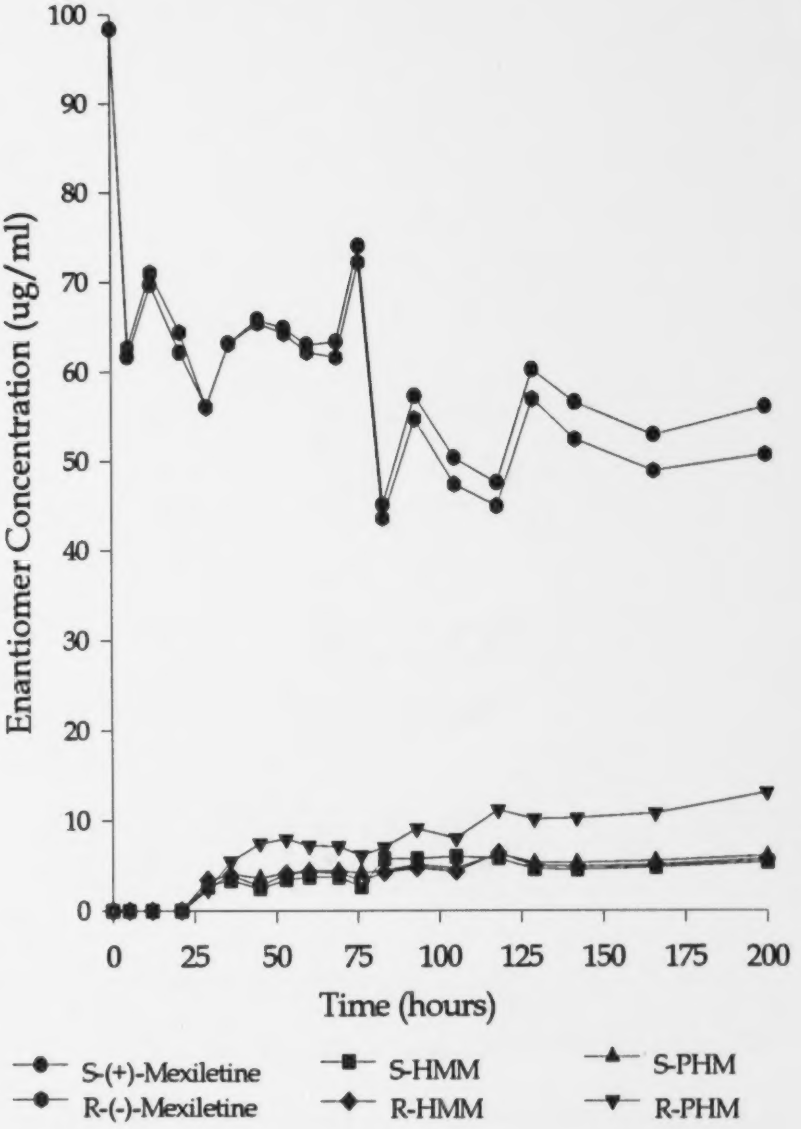


Figure 4-14. A concentration time profile of mexiletine, HMM, and PHM enantiomers in **malt extract broth** (pH 7.0).

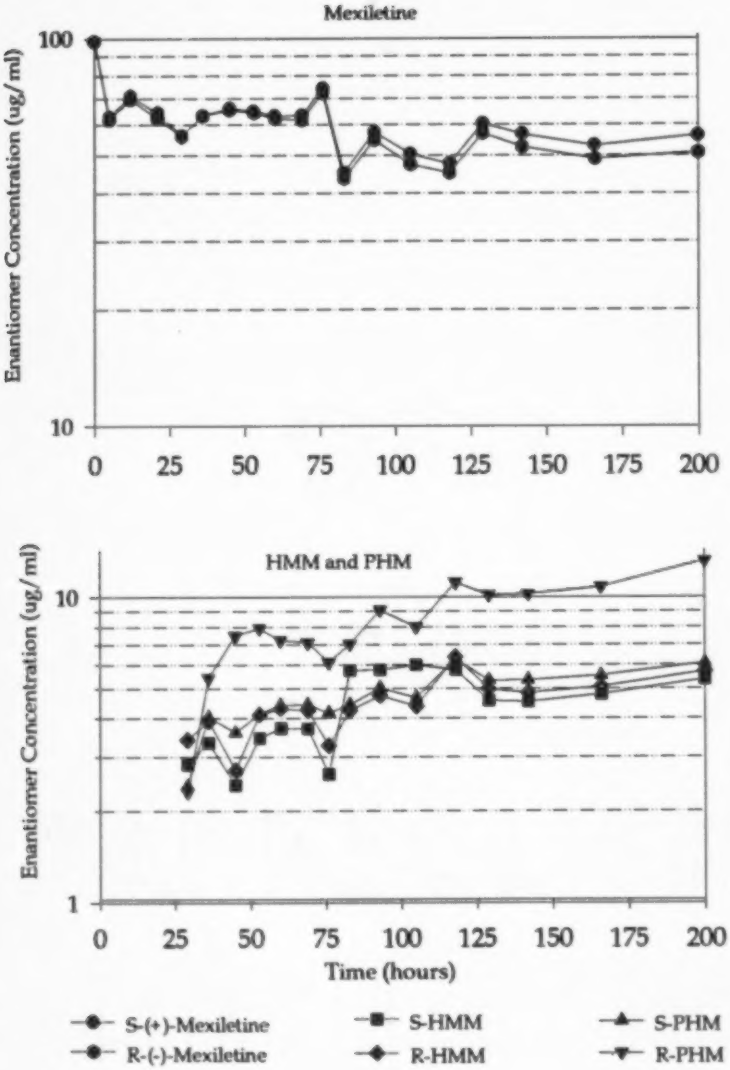


Figure 4-15. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in **malt extract broth** (pH 7.0).

Table 4-13. Metabolism of *rac*-mexiletine in **peptone broth** at pH 7.0.

Average concentration

	Drug Enantiomer Concentration (µg/mL)					
Time	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	54.8	54.6	0.0	0.0	0.0	0.0
12	59.2	55.7	0.0	0.0	0.0	0.0
21	52.1	46.5	2.9	3.2	4.9	8.1
29	49.9	42.6	3.6	4.1	5.6	10.7
36	54.2	44.7	4.7	5.0	6.5	14.7
45	41.3	34.0	4.9	5.1	6.9	15.0
53	47.4	37.6	6.6	6.1	8.0	19.8
60	41.2	31.2	7.2	6.6	8.7	21.9
69	36.3	25.6	7.5	6.9	9.1	23.0
76	34.8	24.3	8.4	7.4	9.9	25.3
83	29.9	20.3	7.9	7.1	9.9	24.5
93	26.8	16.7	7.6	6.8	9.6	23.3
105	26.9	15.6	8.5	7.2	10.9	26.1
118	26.2	14.8	9.4	8.0	12.1	29.8
129	25.8	14.5	10.8	9.4	13.7	34.0
142	25.9	13.8	10.6	8.8	14.0	34.8
166	20.7	10.7	10.4	8.7	14.3	34.6
200	21.0	10.4	11.6	9.4	16.5	39.5

Table 4- 14 A summary of data obtained from the metabolism of *rac*-mexiletine in **peptone broth**.

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	22.3	93	22.7%
R-(-)-Mexiletine	16.1	93	16.4%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	12.1	200	12.3%
R-HMM	9.9	200	10.0%
S-PHM	16.4	200	16.6%
R-PHM	39.2	200	39.9%
Total recovery of added drug (Total amount recovered/amount added x 100			58.9%

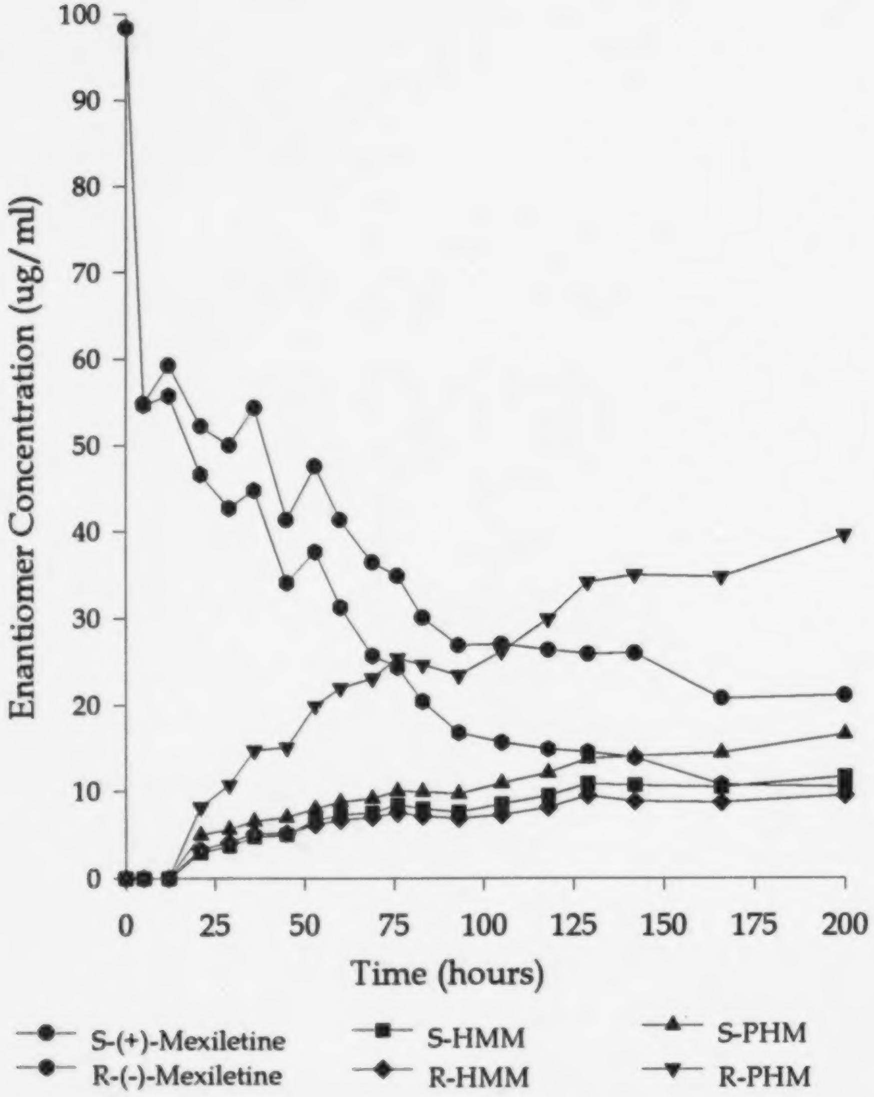


Figure 4-16 A concentration time profile of mexiletine, HMM, and PHM enantiomers in **peptone broth** (pH 7.0).

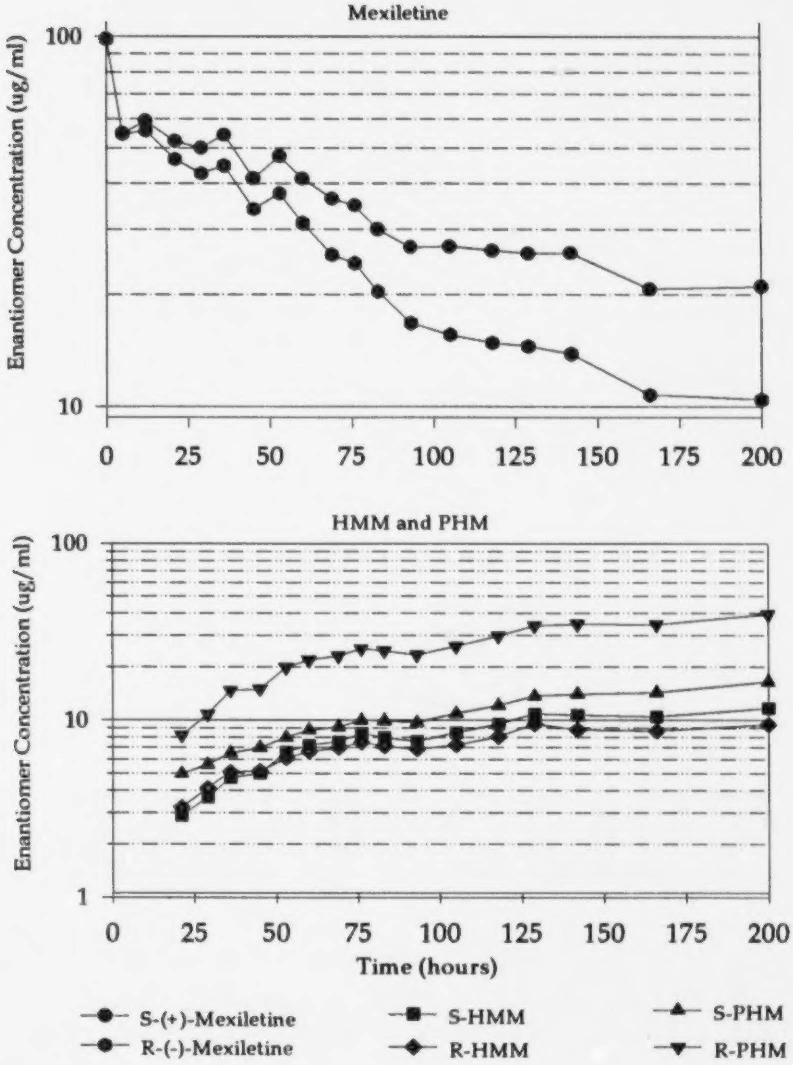


Figure 4-17. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in **peptone broth** (pH 7.0).

Table 4-15. Metabolism of *rac*-mexiletine in **Sabouraud dextrose broth** at pH 7.0.

Average concentration

Time	Drug Enantiomer Concentration (μg/mL)					
	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	98.4	98.4	0.0	0.0	0.0	0.0
5	65.8	67.8	0.0	0.0	0.0	0.0
12	62.0	62.8	1.8	2.1	0.0	2.8
21	53.5	52.1	5.1	2.6	2.6	8.0
29	65.7	62.3	6.0	6.3	6.5	11.0
36	51.9	48.1	7.2	7.5	7.7	16.1
45	46.9	38.7	7.7	7.8	8.4	17.3
53	31.8	25.5	7.4	7.2	7.5	15.8
60	37.6	28.1	8.6	8.4	9.1	20.0
69	28.7	19.4	8.2	7.9	9.1	19.6
76	26.5	18.6	11.7	11.7	12.4	26.6
83	23.9	15.4	9.4	8.8	10.3	22.4
93	23.2	13.7	9.6	8.9	11.0	23.4
105	16.1	9.4	8.5	7.8	9.6	19.8
118	16.8	9.1	10.0	8.5	11.2	24.9
129	15.0	8.4	10.0	8.8	11.3	24.2
142	14.3	7.8	9.4	8.1	10.8	22.0
166	11.2	6.7	9.3	7.6	11.4	24.8
200	9.3	6.3	8.6	6.9	10.4	20.5

Table 4-16. A summary of data obtained from the metabolism of *rac*-mexiletine in **Sabouraud dextrose broth**.

	Minimum conc. remaining (ug/mL).	Time needed to reach minimum conc. (hours).	% of added drug (ug drug remaining/ug added)
S-(+)-Mexiletine	16.2	105	16.4%
R-(-)-Mexiletine	9.4	105	9.6%
	Maximum conc. produced (ug/mL).	Time needed to reach minimum conc. (hours).	% recovery of metabolite (ug drug added/ug metab. formed).
S-HMM	11.8	76	12.0%
R-HMM	11.8	76	12.0%
S-PHM	12.5	76	12.7%
R-PHM	26.6	76	27.1%
Total recovery of added drug (Total amount recovered/amount added x 100)			44.9%

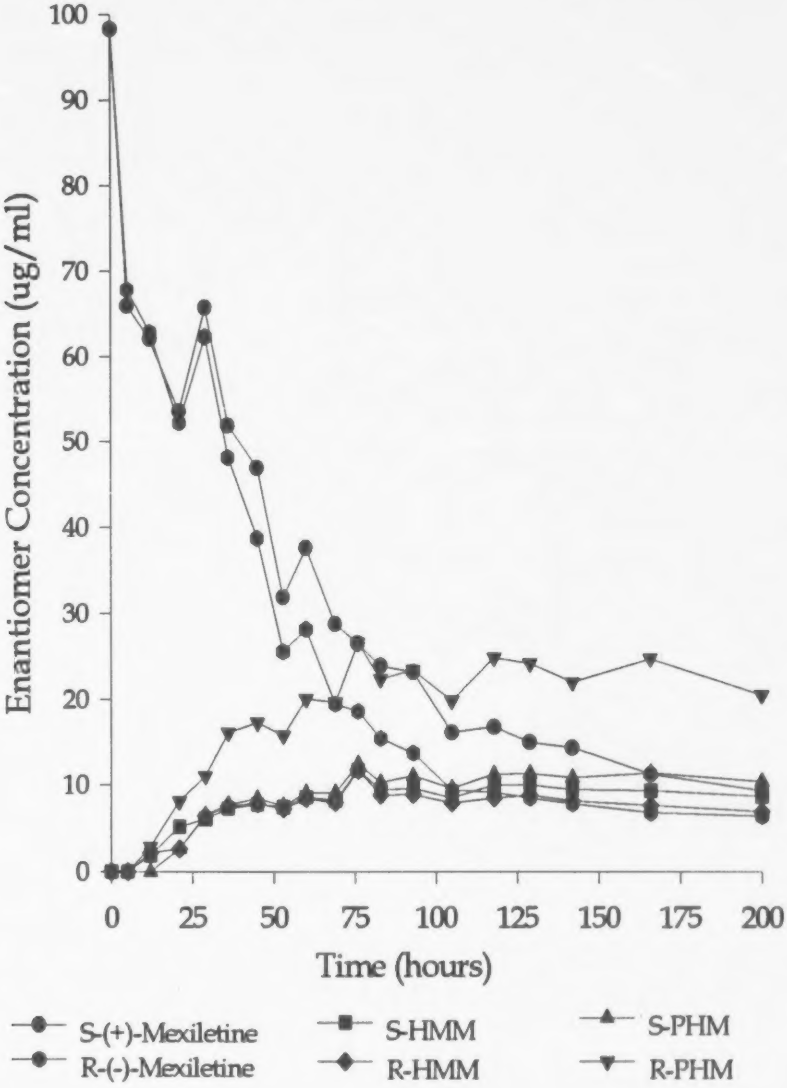


Figure 4-18 A concentration time profile of mexiletine, HMM, and PHM enantiomers in **Sabouraud dextrose broth** (pH 7.0).

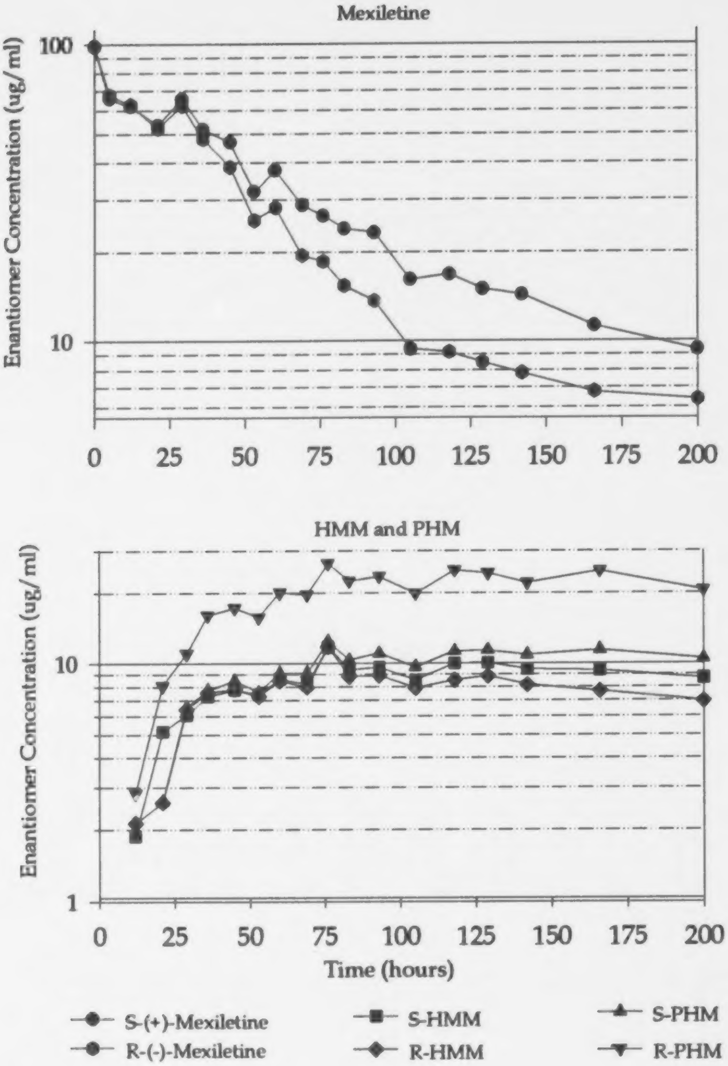


Figure 4-19. A semi-log plot of the concentration time profile of mexiletine, HMM, and PHM enantiomers in **Sabouraud dextrose broth** (pH 7.0).

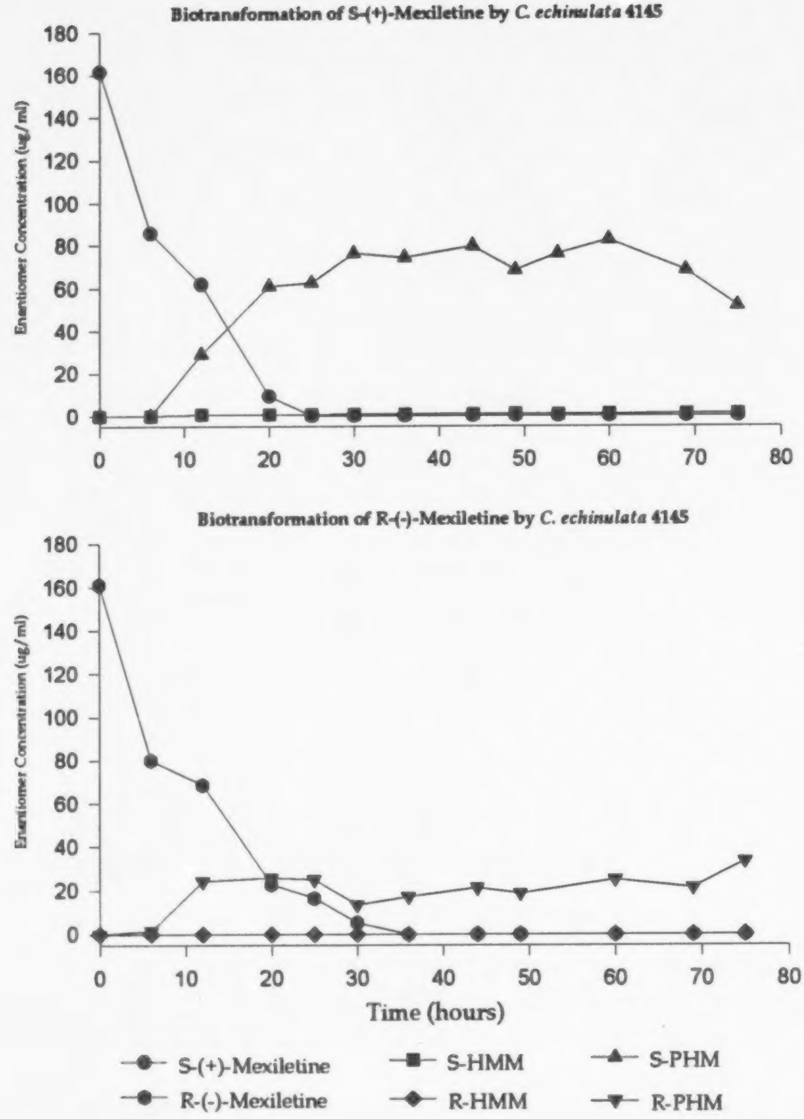
4.2.2 Metabolism of the Individual Enantiomers of Mexiletine

We obtained authentic samples of pure S-(+) and R-(-)-mexiletine from Boehringer Ingelheim, Canada. Therefore it was possible to determine whether the single enantiomers were metabolized differently depending on whether they were incubated as single enantiomers or as a racemate. The media chosen to conduct this experiment were yeast extract and trypticase soy broth. They were chosen because they metabolized *rac*-mexiletine extensively in the previous experiment. This provided a known metabolic profile for comparisons. S-(+)-mexiletine (161.1 μg/mL) and R-(-)-mexiletine (161.5 μg/mL) were aseptically added to each 125 mL Erlenmeyer flask after filter (0.22 μm) sterilization.

Table 4-17. Metabolism of S-(+) and R-(-)-mexiletine in **trypticase soy broth** at pH 7.0.

Time	Drug Enantiomer Concentration (μg/mL)		
(hours)	R-(-)-Mexiletine	(R)-HMM	(R)-PHM
0	161.5	0.0	0.0
6	85.6	0.0	0.0
12	61.8	0.5	29.0
20	9.2	0.6	60.9
25	0.0	0.7	62.4
30	0.0	0.8	76.1
36	0.0	0.8	74.1
44	0.0	0.8	79.5
49	0.0	0.8	68.6
54	0.0	0.8	75.8
60	0.0	0.8	82.4
69	0.0	1.0	68.2
75	0.0	1.0	51.5

Time	Drug Enantiomer Concentration (μg/mL)		
(hours)	S-(+)-Mexiletine	(S)-HMM	(S)-PHM
0	161.1	0.0	0.0
6	79.9	0.0	1.5
12	68.6	0.0	24.2
20	22.7	0.0	25.7
25	16.3	0.0	24.9
30	5.2	0.0	13.4
36	0.0	0.0	17.0
44	0.0	0.0	21.2
49	0.0	0.0	18.6
60	0.0	0.0	24.9
69	0.0	0.0	21.2
75	0.0	0.0	33.4


Biotransformation of S-(+)-Mexiletine by *C. echinulata* 4145

Biotransformation of R-(-)-Mexiletine by *C. echinulata* 4145

Figure 4-20. A concentration time profile of mexiletine, HMM, and PHM enantiomers in trypticase soy broth (pH 7.0).

Table 4-18. Metabolism of S-(+) and R-(-)-mexiletine in yeast extract broth at pH 7.0.

Time	Drug Enantiomer Concentration (μg/mL)		
(hours)	R-(-)-Mexiletine	(R)-HMM	(R)-PHM
0	161.5	0.0	0.0
6	86.1	1.5	3.2
12	88.9	1.4	6.4
20	84.1	1.5	10.7
25	85.0	1.5	16.5
30	81.0	1.6	17.0
36	76.0	1.7	25.6
44	66.1	1.8	28.5
49	57.3	1.7	33.4
54	49.0	1.7	32.2
60	44.1	1.8	36.3
69	34.5	1.8	44.1
75	33.3	2.0	36.4

Time	Drug Enantiomer Concentration (μg/mL)		
(hours)	S-(+)-Mexiletine	(S)-HMM	(S)-PHM
0	161.1	0.0	0.0
6	74.6	1.5	7.1
20	70.8	1.6	17.7
25	49.9	0.9	23.0
30	50.1	1.7	24.7
36	50.4	1.7	25.9
44	40.8	1.9	26.8
49	38.2	1.9	28.7
54	32.8	1.8	23.0
60	39.2	2.0	31.4
69	36.1	2.0	32.1
75	30.0	2.1	30.9

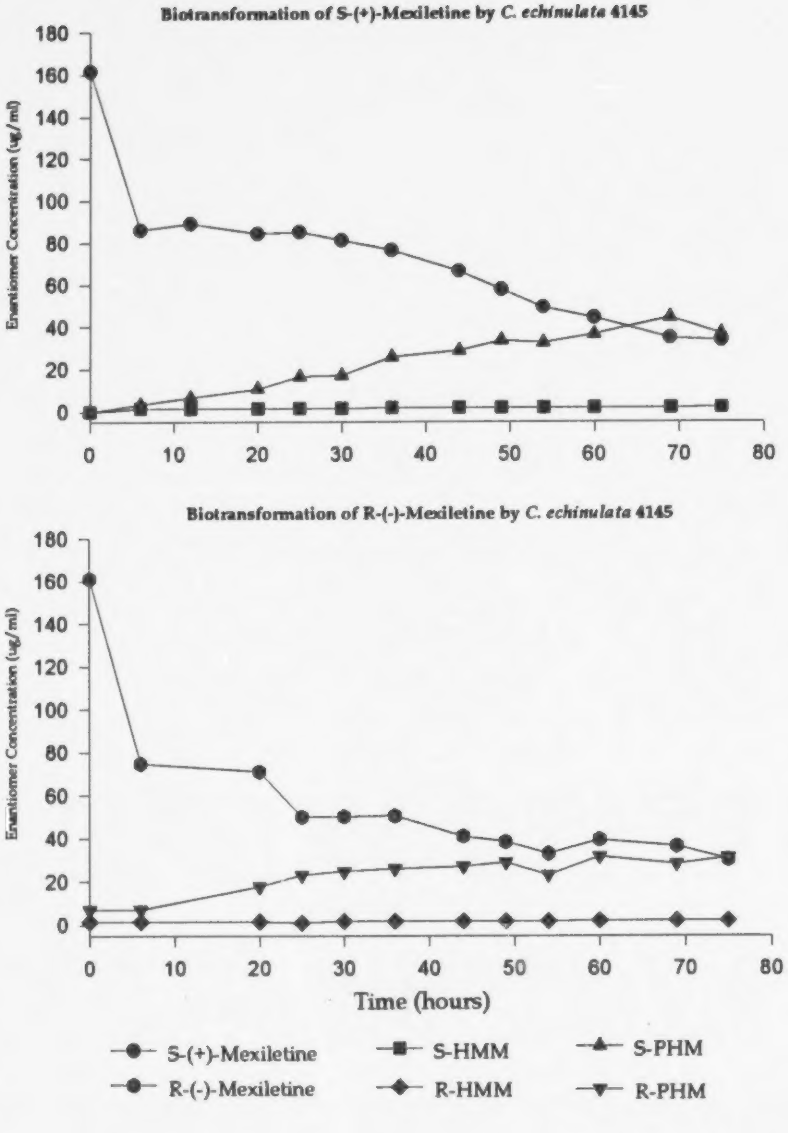


Figure 4-21. A concentration time profile of mexiletine, HMM, and PHM enantiomers in yeast extract broth (pH 7.0).

4.2.3 Effect of pH on the Biosynthesis of HMM and PHM

Another growth parameter which required consideration was medium pH. Previous biotransformation experiments were arbitrarily carried out at pH 7.0. However, we were uncertain whether this was the optimal pH for metabolite biosynthesis. The media formulations tested (yeast extract, tripticase soy, peptone, malt extract, and Sabauroud dextrose broth) were ones that yielded the most HMM and PHM. These experiments were carried out in 125 mL Erlenmeyer flasks, containing 25 mL of medium, using a racemic drug concentration of 205.9 μg/mL. The concentration was determined from HPLC analysis of the sterile stock solution. The recorded pH and media formulations used are listed in the following tables and graphs (Figures 4-22 to 4-26, and Tables 4-19, to 4-26).

The following buffers were used to maintain media pH:

pH 5.0 - 100 mL of a sodium acetate/acetic acid buffer was prepared using:

Solution A: 8.2 g/L sodium acetate.
Solution B: 6.0 g/L acetic acid.

Combine 67.8 mL of solution A and 32.2 mL of solution B

pH 5.5, 6.0, and 6.5 - Tris maleate/sodium hydroxide buffer was prepared by:

Add 1.52 g Tris and 1.46 g maleic acid to 1.0 L of water and bring to the pH using 5.0 M NaOH.

pH 8.0 - A Tris and hydrochloric acid buffer was prepared.

A 1.0 M Tris solution was prepared and adjusted to pH 8.0 using concentrated HCl.

After the buffers had been prepared, dry media components were added as indicated in the materials and methods section.

No growth was observed in flasks containing yeast extract broth and peptone broth at a pH of 5.0.

Table 4-19. Metabolism of *rac*-mexiletine in yeast extract broth at the initial pH indicated.

Initial pH of 5.5

Time (hours)	Drug Enantiomer Concentration (μg/mL)						pH During Incubation
	Mexiletine		HMM		PHM		
	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	5.50
2	63.6	63.5	0.0	0.0	0.0	0.0	5.54
4	36.0	35.9	0.0	0.0	0.0	0.0	
8	47.8	48.1	0.0	0.0	0.0	0.0	
12	61.2	61.9	0.0	0.0	0.0	0.0	5.04
21	59.7	60.0	0.0	0.0	0.0	0.0	5.07
28	53.4	53.5	0.0	0.0	0.0	0.0	
36	27.5	25.7	3.2	4.1	7.3	9.8	6.79
50	25.7	20.1	6.1	6.8	10.8	20.3	7.55
60	14.1	12.1	4.5	5.5	9.9	16.7	7.88

Initial pH of 6.0

Time (hours)	Drug Enantiomer Concentration (μg/mL)						pH During Incubation
	Mexiletine		HMM		PHM		
	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.00	0.0	6.00
2	63.1	63.1	0.0	0.0	0.0	0.0	5.89
4	30.7	31.1	0.0	0.0	0.00	0.0	
8	53.3	53.8	0.0	0.00	0.0	0.0	
12	60.5	61.4	0.0	0.0	0.0	0.0	5.44
21	66.6	67.2	0.0	0.0	0.0	0.0	5.56
28	60.7	60.3	2.9	3.7	6.9	8.6	
36	33.5	31.1	3.5	4.3	7.6	11.3	6.81
50	33.2	23.9	6.9	6.7	11.5	22.0	7.51
60	18.9	14.4	5.8	5.8	10.8	20.0	7.90

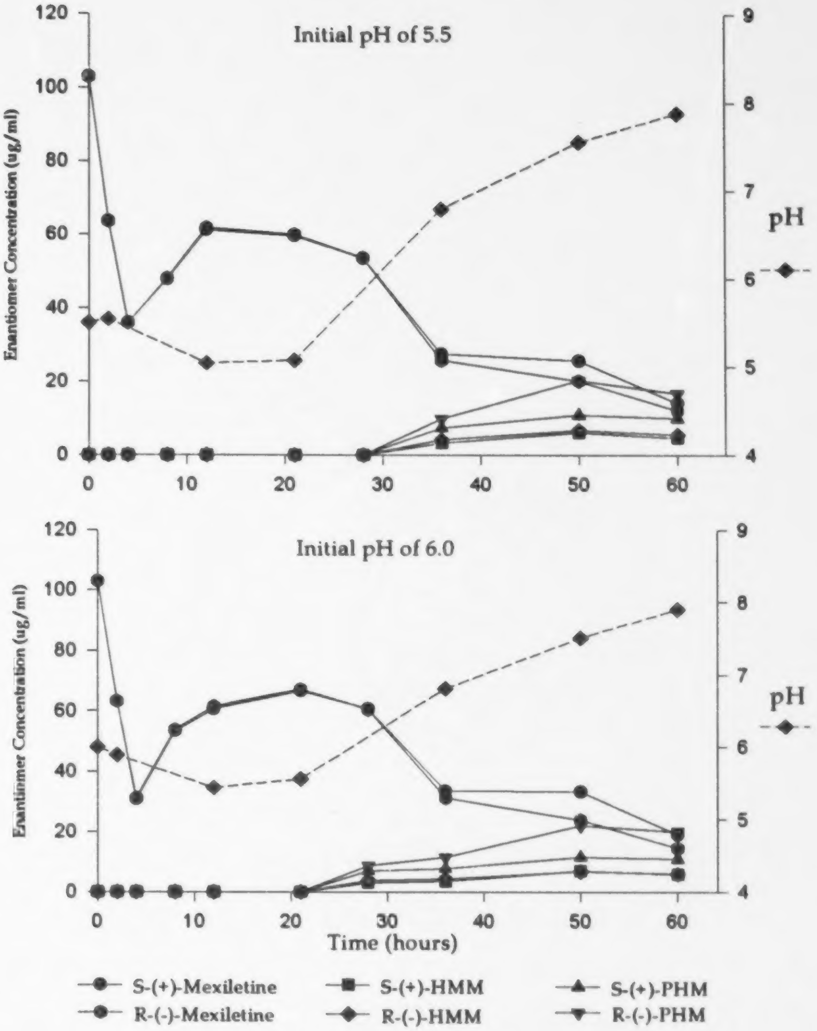


Figure 4-22. A concentration time profile of *rac*-mexiletine, HMM, and PHM in **yeast extract broth at pH 5.5 and 6.0.**

Table 4-20. Metabolism of *rac*-mexiletine in **yeast extract broth** at the initial pH indicated.

Initial pH of 6.5

	Drug Enantiomer Concentration (µg/mL)						pH During Incubation
Time	Mexiletine		HMM		PHM		
(hours)	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	6.50
2	63.3	64.0	0.0	0.0	0.0	0.0	6.22
4	59.2	59.8	0.0	0.0	0.0	0.0	
8	62.2	62.0	0.0	0.0	0.0	0.0	
12	67.5	68.3	0.0	0.0	0.0	0.0	5.95
21	59.1	58.9	2.8	3.6	7.0	7.8	5.99
28	57.5	56.2	3.0	4.0	7.4	9.4	
36	53.4	49.5	4.4	4.9	8.4	13.7	6.80
50	30.4	23.2	7.3	7.0	11.4	24.2	7.31
60	19.6	13.7	6.7	6.1	11.2	20.3	7.77

Initial pH of 8.0

	Drug Enantiomer Concentration (µg/mL)						pH During Incubation
Time	Mexiletine		HMM		PHM		
(hours)	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	8.00
2	46.6	46.8	0.0	0.0	0.0	0.0	7.65
4	39.7	39.2	0.0	0.0	0.0	0.0	
8	42.9	43.3	0.0	0.0	0.0	0.0	
12	29.6	29.7	0.0	0.0	0.0	0.0	7.72
21	43.3	43.7	0.0	0.0	0.0	0.0	7.74
28	40.2	40.7	0.0	0.0	0.0	0.0	
36	39.6	39.9	0.0	0.0	0.0	0.0	7.75
50	40.3	40.8	0.0	0.0	0.0	0.0	7.78
60	34.4	34.9	0.0	0.0	0.0	0.0	8.03

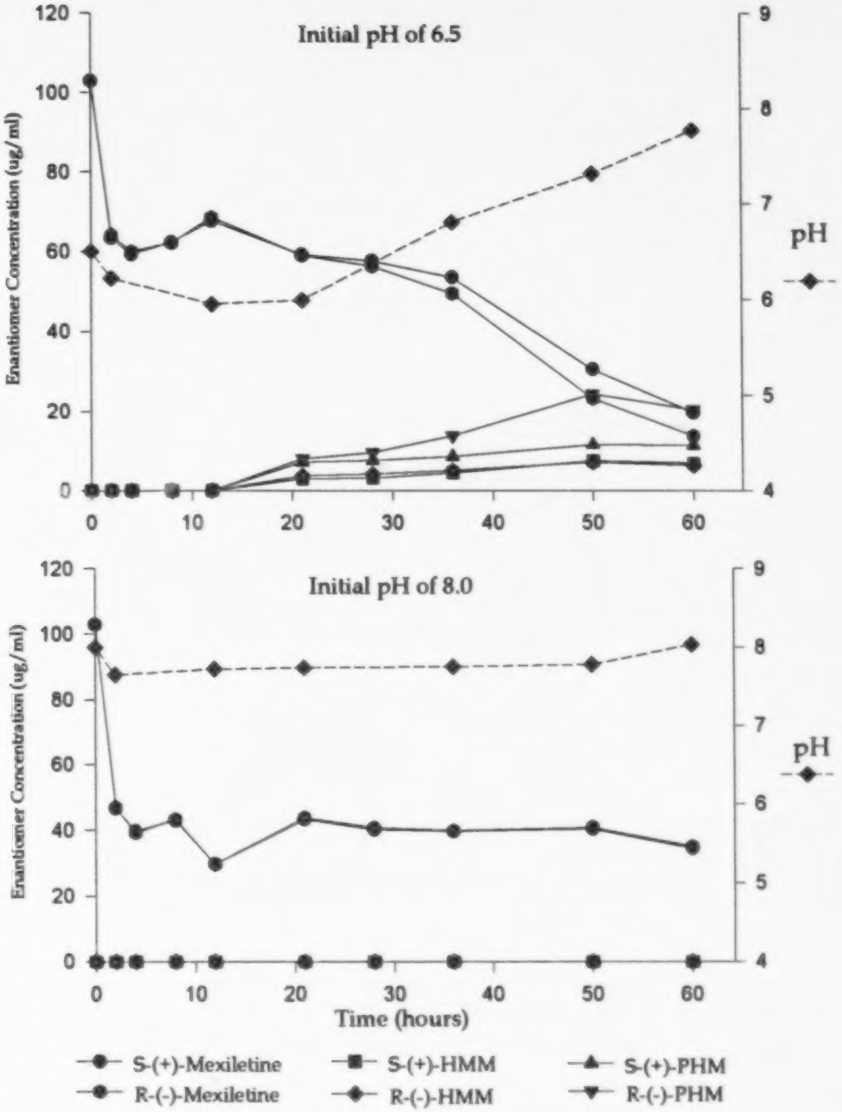


Figure 4-23. A concentration time profile of *rac*-mexiletine, HMM, and PHM in yeast extract broth at pH 6.5 and 8.0.

Table 4-21. Metabolism of *rac*-mexiletine in **trypticase soy broth** at the initial pH indicated.

Initial pH of 5.0

	Drug Enantiomer Concentration (µg/mL)						**pH** During Incubation
Time	Mexiletine		HMM		PHM		
(hours)	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	5.00
2	65.1	66.1	0.0	0.0	0.0	0.0	5.71
4	60.4	61.4	0.0	0.0	0.0	0.0	
8	57.5	58.7	0.0	0.0	0.0	0.0	
12	62.7	63.8	0.0	0.0	0.0	0.0	5.72
21	50.5	51.3	0.0	0.0	0.0	0.0	6.86
28	52.0	47.4	6.7	6.7	9.8	17.0	
36	37.9	31.5	7.9	8.1	11.4	22.4	8.33
50	40.0	32.9	8.5	7.9	11.3	23.4	8.61
60	36.6	30.2	8.5	8.0	11.6	23.5	8.89

Initial pH of 5.5

	Drug Enantiomer Concentration (µg/mL)						**pH** During Incubation
Time	Mexiletine		HMM		PHM		
(hours)	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	5.50
2	32.7	33.1	0.0	0.0	0.0	0.0	7.01
4	37.6	38.2	0.0	0.0	0.0	0.0	
8	44.1	44.3	4.2	4.3	0.0	5.4	
12	38.2	35.2	7.6	6.9	6.6	15.7	7.61
21	19.0	14.9	8.4	7.3	10.9	21.0	8.01
28	18.1	13.5	9.4	8.5	12.8	25.0	
36	15.5	11.8	7.8	6.6	12.1	23.8	8.32
50	13.0	10.3	6.5	5.5	11.2	19.8	8.35
60	11.0	9.3	5.5	5.0	9.5	13.7	8.68

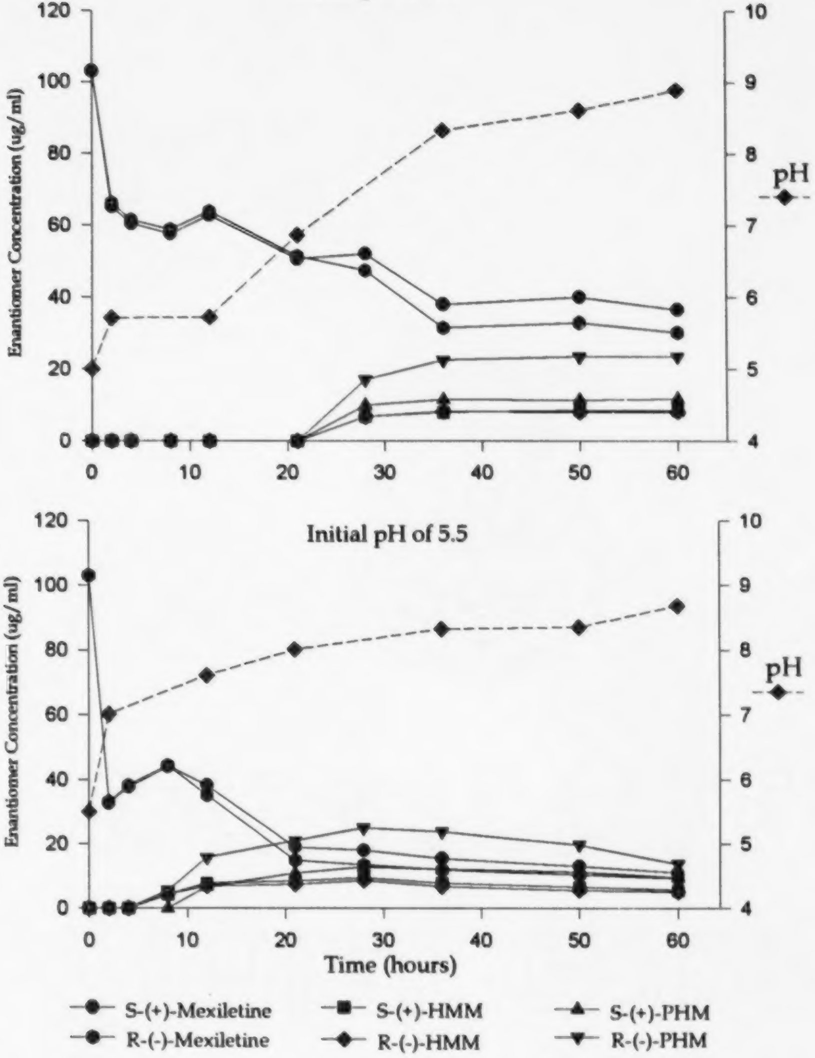


Figure 4-24. A concentration time profile of *rac*-mexiletine, HMM, and PHM in **trypticase soy broth** at pH 5.0 and 5.5.

Table 4-22. Metabolism of *rac*-mexiletine in **trypticase soy broth** at the initial pH indicated.

Initial pH of 6.0

Time (hours)	Drug Enantiomer Concentration (µg/mL)						**pH** During Incubation
	Mexiletine		HMM		PHM		
	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	6.00
2	43.3	43.9	0.0	0.0	0.0	0.0	7.16
4	24.0	24.0	0.0	0.0	0.0	0.0	
8	48.3	47.9	4.2	4.3	7.0	7.5	
12	40.5	36.7	11.2	10.8	12.1	18.1	7.65
21	16.3	13.	5.6	5.4	8.8	13.5	7.98
28	15.3	12.3	5.8	5.7	9.5	15.0	
36	14.5	11.9	6.1	5.6	10.7	18.5	8.29
50	14.9	11.5	7.5	6.2	12.4	24.5	8.37
60	12.1	10.1	6.1	5.6	11.1	19.7	8.77

Initial pH of 6.5

Time (hours)	Drug Enantiomer Concentration (µg/mL)						**pH** During Incubation
	Mexiletine		HMM		PHM		
	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	6.50
2	38.1	38.5	0.0	0.0	0.0	0.0	7.30
4	31.9	32.5	0.00	0.0	0.0	0.0	
8	37.0	36.7	4.2	4.3	7.1	7.5	
12	25.0	22.3	5.2	5.0	8.3	11.4	7.73
21	17.7	14.3	6.6	6.1	10.4	19.2	8.16
28	17.9	13.7	8.0	6.6	12.3	25.3	
36	13.8	11.1	6.7	6.0	10.7	20.1	8.38
50	13.7	10.7	7.5	6.4	12.7	25.7	8.43
60	12.3	10.0	6.8	6.1	11.9	23.4	8.92

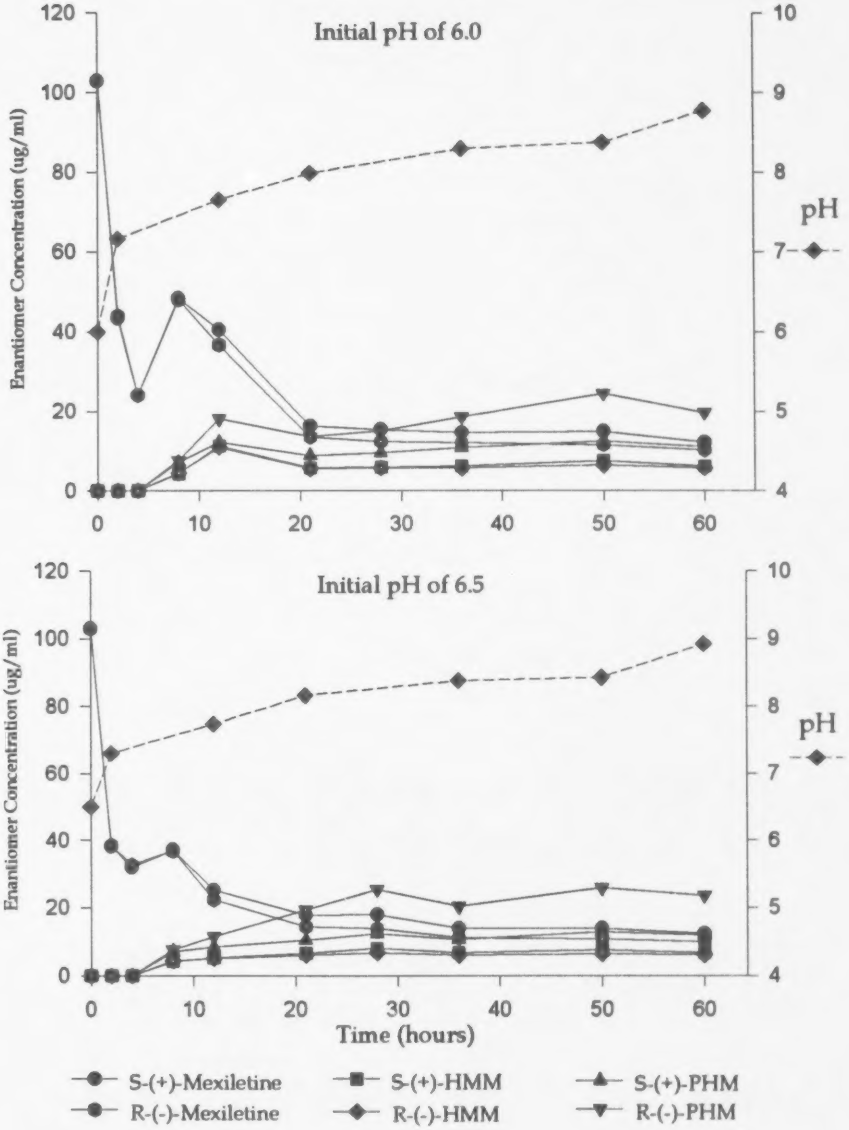


Figure 4-25. A concentration time profile of *rac*-mexiletine, HMM, and PHM in **trypticase soy broth** at pH 6.0 and 6.5.

Table 4-23. Metabolism of *rac*-mexiletine in **trypticase soy broth** at the initial pH indicated.

Initial pH of 8.0

Time	Drug Enantiomer Concentration (µg/mL)						**pH** During Incubation
	Mexiletine		HMM		PHM		
(hours)	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	8.00
2	22.4	22.5	0.0	0.0	0.0	0.0	7.83
4	26.5	26.5	0.0	0.0	0.0	0.0	
8	26.4	26.2	0.0	0.0	0.0	0.0	
12	24.1	24.3	0.0	0.0	0.0	0.0	7.92
21	44.6	45.1	0.0	0.0	0.0	0.0	7.92
28	44.6	45.1	0.0	0.0	0.0	0.0	
36	40.1	40.5	0.0	0.0	0.0	0.0	7.92
50	39.9	40.2	0.0	0.0	0.0	0.0	7.92
60	45.8	46.2	0.0	0.0	4.7	10.3	8.19

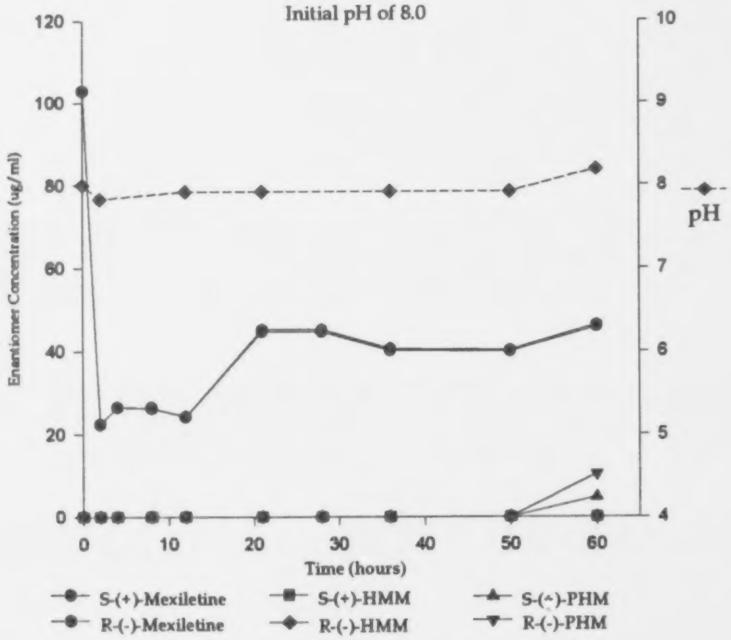


Figure 4-26. A concentration time profile of *rac*-mexiletine, HMM, and PHM in **trypticase soy broth** at pH 8.0.

Table 4-24. Metabolism of *rac*-mexiletine in **peptone broth** at the initial pH indicated.

Initial pH of 5.5

Time (hours)	Drug Enantiomer Concentration (μg/mL)						**pH During Incubation**
	Mexiletine		HMM		PHM		
	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	5.50
2	64.8	65.7	0.0	0.0	0.0	0.0	5.42
4	60.9	61.7	0.0	0.0	0.0	0.0	
8	55.3	56.1	0.0	0.0	0.0	0.0	
12	57.9	58.8	0.0	0.0	0.0	0.0	5.22
21	68.2	69.4	0.0	0.0	0.0	0.0	5.04
28	51.5	52.1	0.0	0.0	0.0	0.0	
36	58.0	58.8	0.0	0.0	0.0	0.0	5.36
50	53.5	54.0	0.0	0.0	0.0	0.0	6.34
60	50.1	49.6	9.6	9.6	10.8	12.1	6.82

Initial pH of 6.0

Time (hours)	Drug Enantiomer Concentration (μg/mL)						**pH During Incubation**
	Mexiletine		HMM		PHM		
	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	6.00
2	53.0	53.4	0.0	0.0	0.0	0.0	5.68
4	53.1	53.8	0.0	0.0	0.0	0.0	
8	52.7	53.7	0.0	0.0	0.0	0.0	
12	54.8	55.9	0.0	0.0	0.0	0.0	5.52
21	58.5	59.3	0.0	0.0	0.0	0.0	5.40
28	46.6	47.0	0.0	0.0	0.0	0.0	
36	51.2	51.9	0.0	0.0	0.0	0.0	5.67
50	48.2	48.8	9.1	9.2	0.0	10.6	6.39
60	37.1	36.7	9.3	9.4	10.4	11.4	6.80

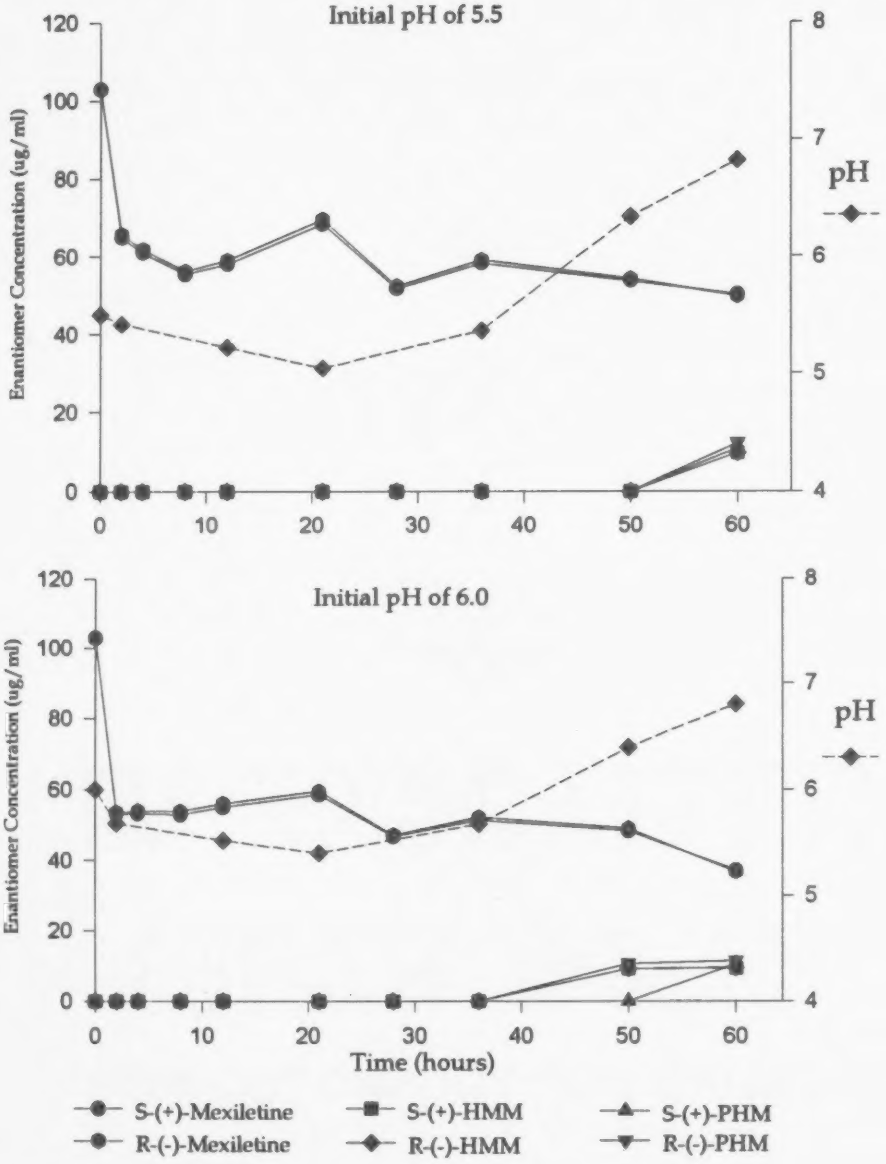


Figure 4-27. A concentration time profile of *rac*-mexiletine, HMM, and PHM in **peptone broth** at pH 5.5 and 6.0.

Table 4-25. Metabolism of *rac*-mexiletine in **peptone broth** at the pH indicated.

Initial pH of 6.5

	Drug Enantiomer Concentration (μg/mL)						**pH** During Incubation
Time	Mexiletine		HMM		PHM		
(hours)	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	6.50
2	55.1	55.9	0.0	0.0	0.0	0.0	6.08
4	47.3	48.3	0.0	0.0	0.0	0.0	
8	42.8	43.6	0.0	0.0	0.0	0.0	
12	53.8	54.7	0.0	0.0	0.0	0.0	5.77
21	56.6	57.5	0.0	0.0	0.0	0.0	5.60
28	46.7	47.4	0.0	0.0	0.0	0.0	
36	40.7	41.3	0.0	0.0	0.0	0.0	5.93
50	48.2	48.4	0.0	0.0	0.0	0.0	6.58
60	41.7	41.3	9.5	9.3	10.1	11.2	6.92

Initial pH of 8.0

	Drug Enantiomer Concentration (μg/mL)						**pH** During Incubation
Time	Mexiletine		HMM		PHM		
(hours)	S-(+)	R-(-)	S	R	S	R	
0	102.9	102.9	0.0	0.0	0.0	0.0	8.00
2	39.9	40.3	0.0	0.0	0.0	0.0	7.82
4	45.8	46.4	0.0	0.0	0.0	0.0	
8	40.7	41.2	0.0	0.0	0.0	0.0	
12	46.4	46.8	0.0	0.0	0.0	0.0	7.86
21	46.0	46.6	0.0	0.0	0.0	0.0	7.91
28	41.0	41.4	0.0	0.0	0.0	0.0	
36	37.3	37.7	0.0	0.0	0.0	0.0	7.91
50	32.4	32.8	0.0	0.0	0.0	0.0	7.91
60	38.4	38.7	0.0	0.0	0.0	0.0	8.04

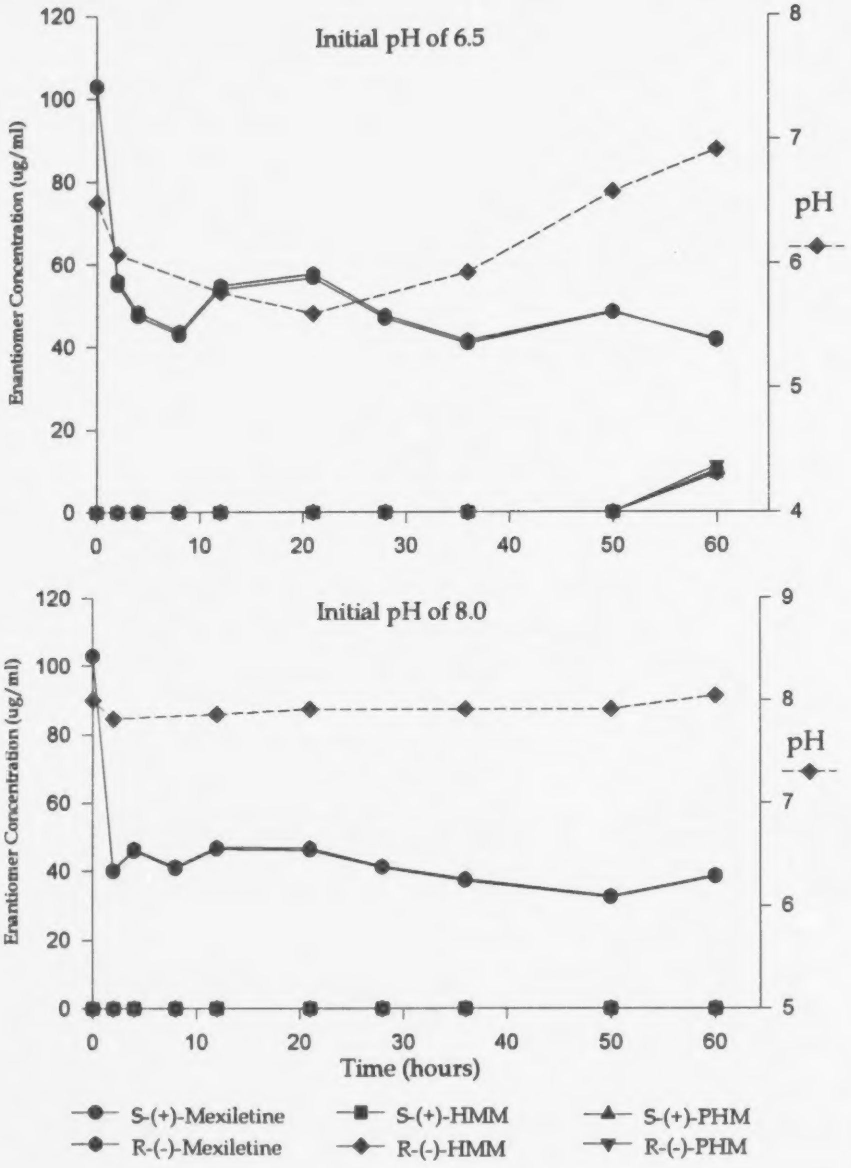


Figure 4-28. A concentration time profile of *rac*-mexiletine, HMM, and PHM in peptone broth at pH 6.5 and 8.0.

Table 4-26. Metabolism of *rac*-mexiletine using **malt extract broth**, at pH 4.7, and **Sabouraud dextrose broth** at pH 5.7.

Initial pH 4.7

Malt Extract Broth							
	Drug Enantiomer Concentration (µg/mL)						**pH**
Time	Mexiletine		HMM		PHM		During
(hours)	S-(+)	R-(-)	S	R	S	R	Incubation
0	102.9	102.9	0.0	0.0	0.0	0.0	5.50
2	60.2	61.1	0.0	0.0	0.0	0.0	5.12
4	47.4	48.5	0.0	0.0	0.0	0.0	
8	73.9	75.6	0.0	0.0	0.0	0.0	
12	57.9	59.1	0.0	0.0	0.0	0.0	4.73
21	66.8	68.1	0.0	0.0	0.0	0.0	4.72
28	58.7	59.7	0.0	0.0	0.0	0.0	
36	55.2	56.2	0.0	0.0	0.0	0.0	4.63
50	57.7	59.1	0.0	0.0	0.0	0.0	4.72
60	49.4	49.7	0.0	0.0	0.0	0.0	4.45

Initial pH 5.7

Sabouraud Dextrose Broth							
	Drug Enantiomer Concentration (µg/mL)						**pH**
Time	Mexiletine		HMM		PHM		During
(hours)	S-(+)	R-(-)	S	R	S	R	Incubation
0	102.9	102.9	0.0	0.0	0.0	0.0	5.70
2	40.1	40.6	0.0	0.0	0.0	0.0	5.47
4	32.5	32.8	0.0	0.0	0.0	0.0	
8	41.6	42.4	0.0	0.0	0.0	0.0	
12	28.1	28.2	0.0	0.0	0.0	0.0	4.99
21	38.6	39.1	0.0	0.0	0.0	0.0	4.42
28	31.3	31.4	0.0	0.0	0.0	0.0	
36	35.9	36.8	0.0	0.0	0.0	0.0	4.28
50	25.1	25.4	0.0	0.0	0.0	0.0	7.01
60	17.2	15.5	4.7	4.3	0.0	11.2	7.65

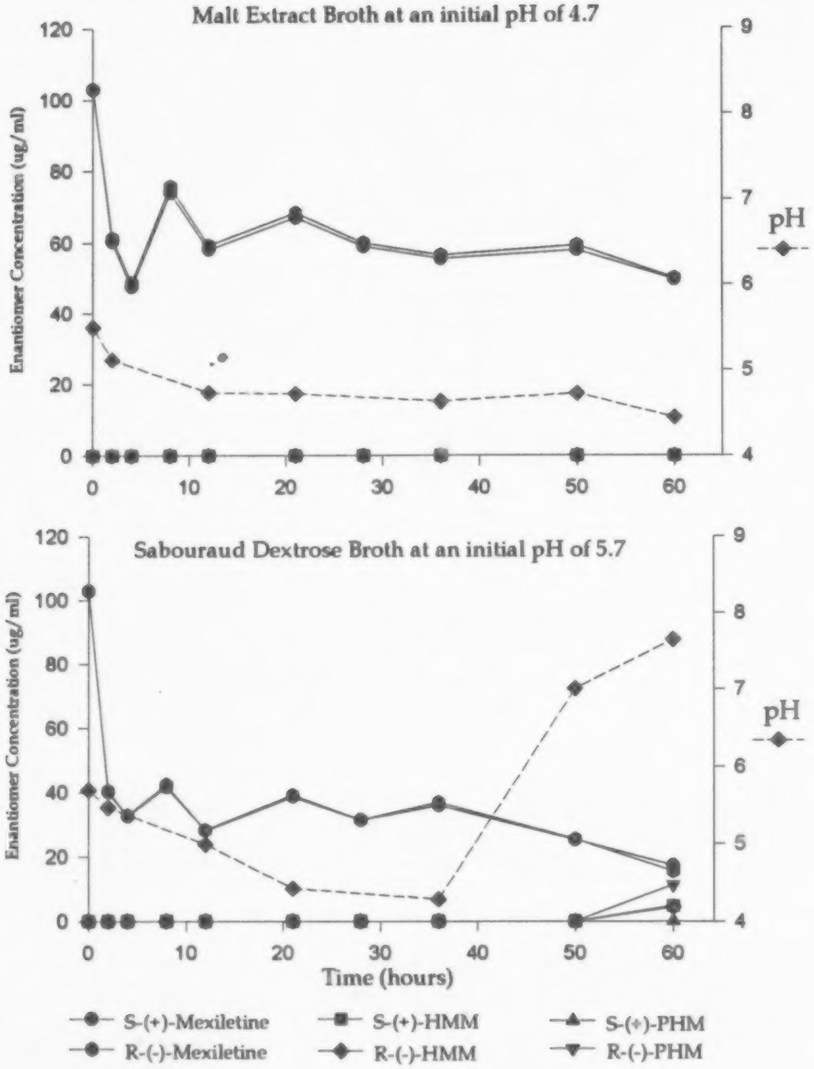


Figure 4-29. A concentration time profile of *rac*-mexiletine, HMM, and PHM in **malt extract broth**, at pH 4.7, and **Sabouraud dextrose broth** at pH 5.7.

4.2.5 Biosynthesis of Acetylated Phase II Metabolites

In the biotransformation experiments discussed to this point, the maximum recovery of *rac*-mexiletine, HMM, and PHM in total was 71% (casamino acid broth). Therefore, a significant amount of drug was unaccounted for. However, there are reports of *Cunninghamella's* ability to biosynthesize phase II metabolites as a way of eliminating (detoxifying) foreign compounds. Foster *et al.* (1989) reported that the fungus *Cunninghamella bainieri* was capable of acetylating the amino group of aryl alkylamines. Considering *rac*-mexiletine has a similar structure, it was feasible that unaccounted drug was being transformed into acetylated phase II metabolites. In order to determine this, an HPLC assay was developed (see methods and materials).

Results from this study are listed below. Samples collected and stored at -20° C from the previous nitrogen experiments (section 4.2.1) were re-tested for the presence of acetylmexiletine, acetyl-HMM, and acetyl-PHM. Confirmation of the identity of these metabolites was made by comparing retention times with standards synthesized in our laboratory.

Table 4-27. Analysis of **trypticase soy broth** for the production of acetylated, phase II metabolites from *rac*-mexiletine.

Cunninghamella echinulata 4145			
Time (hours)	Concentration of Compound (μg/mL)		
	Acetylmexiletine	Acetyl-HMM	Acetyl-PHM
0	0.0	0.0	0.0
5	0.0	0.0	2.8
12	0.0	0.0	2.8
21	2.4	0.0	4.6
29	3.3	1.4	5.2
45	4.1	1.4	6.4
53	4.3	2.1	8.3
60	3.8	2.2	8.4
69	3.1	1.5	7.5
76	3.3	1.6	8.2
83	3.8	1.5	8.2
93	2.8	1.5	7.8
105	3.0	1.5	8.4
118	3.1	1.6	8.6

Table 2-27 Continued

Time (h)	Acetylmexiletine	Acetyl-HMM	Acetyl-PHM
129	3.3	1.6	8.9
142	2.7	1.5	7.8
166	3.8	1.8	9.2
200	3.1	1.5	8.5

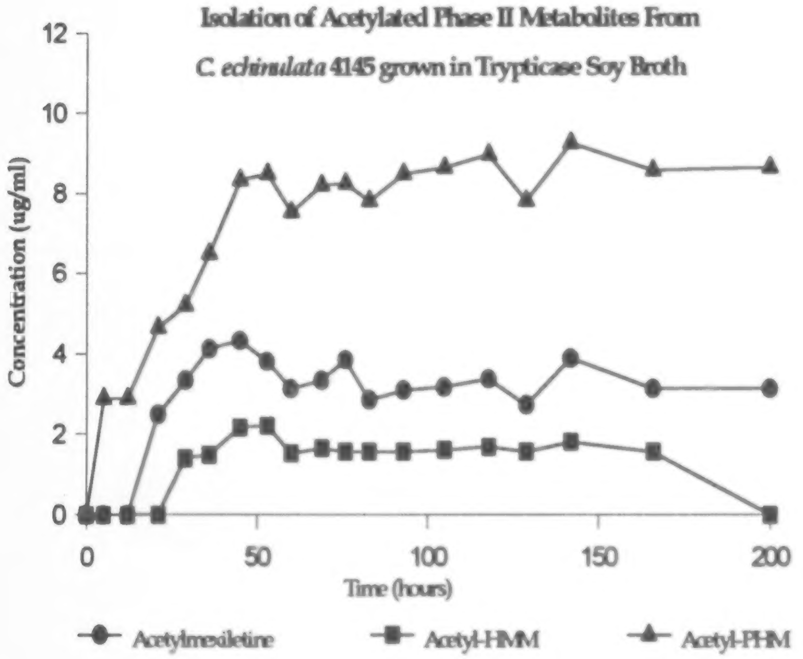


Figure 4-30. A concentration time profile of acetylmexiletine, acetyl-HMM, and acetyl PHM biosynthesis in **trypticase soy broth**.

Table 4-28. Analysis of **phosphate buffer** for the production of acetylated phase II metabolites from *rac*-mexiletine.

Cunninghamella echinulata 4145 Grown in: Phase I - Trypticase Broth Phase II - Phosphate Buffer			
Time (hours)	Concentration of Compound (μg/mL)		
	Acetylmexiletine	Acetyl-HMM	Acetyl-PHM
0	0.0	0.0	0.0
5	0.0	0.0	3.2
12	0.0	0.0	3.3
21	0.0	0.0	3.2
29	0.0	0.0	2.9
36	0.5	0.0	3.5
45	1.7	0.0	3.7
53	1.2	0.0	3.4
60	2.2	0.0	3.6
69	2.6	0.0	3.6
76	2.5	0.0	3.8
83	2.2	0.0	3.4
93	4.3	0.0	3.9
105	4.4	0.0	3.9
118	4.0	0.0	4.1
129	4.3	0.0	4.2
142	4.6	0.0	4.6
166	5.0	0.0	4.3
200	5.4	0.0	4.3

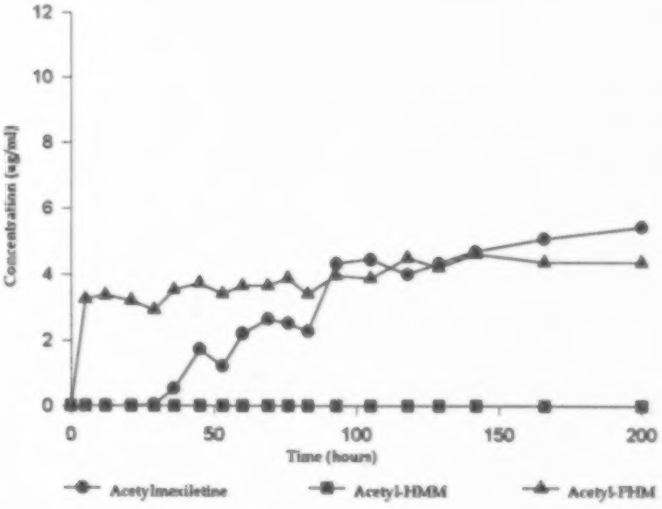


Figure 4-31. A concentration time profile of acetylmexiletine, acetyl-HMM, and acetyl PHM biosynthesis in trypticase soy broth (phase I) and **phosphate buffer** (phase II).

Table 4-29. Analysis of **yeast extract broth** for the production of acetylated phase II metabolites from *rac*-mexiletine.

Cunninghamella echinulata 4145			
Time	Concentration of Compound (µg/mL)		
(hours)	Acetylmexiletine	Acetyl-HMM	Acetyl-PHM
0	0.0	0.0	0.0
5	0.0	0.4	2.0
12	0.1	0.5	1.8
21	0.2	0.5	1.6
29	0.4	0.4	1.8
45	0.4	0.8	1.9
53	0.4	0.5	1.4
60	0.4	0.5	1.2
69	0.3	0.7	1.4
76	0.4	0.5	1.4
83	0.4	0.6	1.6
93	0.4	0.7	1.6
105	0.4	0.7	2.4
118	0.3	1.0	1.9
129	0.2	0.8	1.9
142	0.2	0.6	1.4
166	0.0	0.0	2.2
200	0.0	0.0	1.7

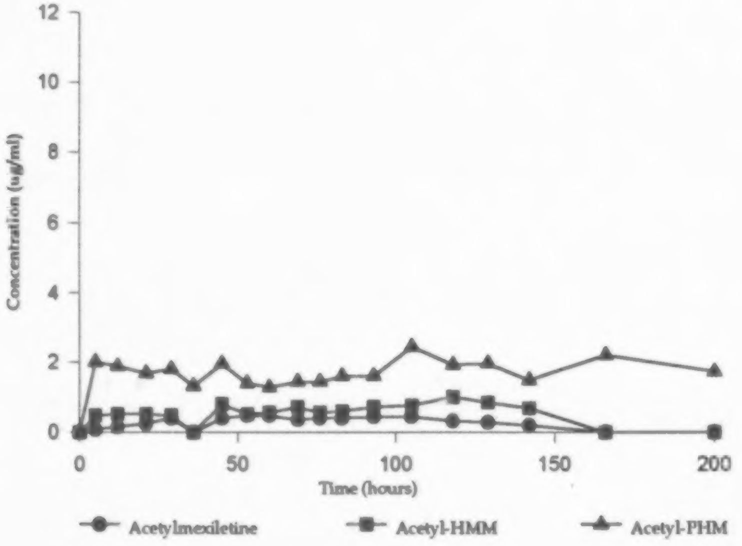


Figure 4-32. A concentration time profile of acetylmexiletine, acetyl-HMM, and acetyl PHM biosynthesis in **yeast extract broth**.

Table 4-30. Analysis of **peptone broth** for the production of acetylated phase II metabolites from *rac*-mexiletine.

Cunninghamella echinulata 4145			
Time	Concentration of Compound (μg/mL)		
(hours)	Acetylmexiletine	Acetyl-HMM	Acetyl-PHM
0	0.0	0.0	0.0
5	0.0	0.8	2.0
12	4.8	0.6	1.9
21	4.9	0.5	2.1
29	4.9	0.5	2.5
36	4.9	0.5	1.6
45	5.2	0.5	1.6
53	4.6	0.5	1.5
60	4.9	0.5	1.6
76	4.9	0.4	2.2
83	5.0	0.4	1.5
93	5.0	0.3	2.0
105	4.9	0.4	1.9
118	5.0	0.4	1.9
129	4.9	0.3	2.1
142	5.1	0.0	2.1
166	4.6	0.0	2.7
200	4.6	0.0	2.5

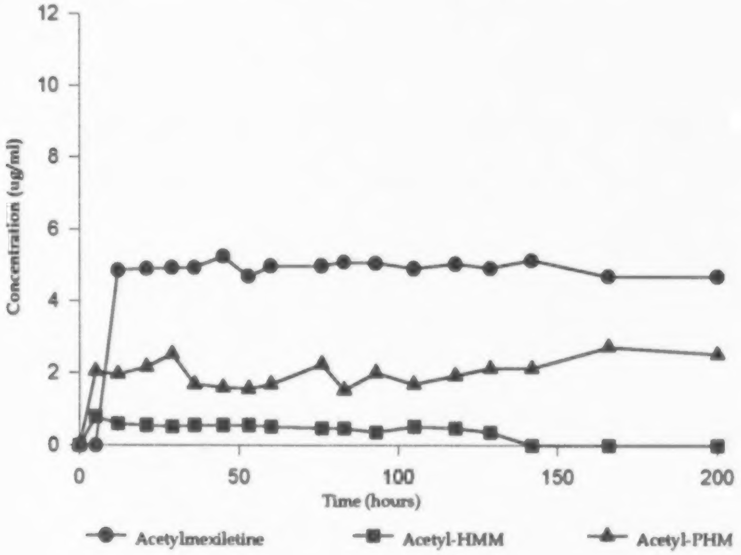


Figure 4-33. A concentration time profile of acetylmexiletine, acetyl-HMM, and acetyl PHM biosynthesis in **peptone broth**.

Table 4-31. Analysis of **czapec dox broth** for the production of acetylated phase II metabolites from *rac*-mexiletine.

Cunninghamella echinulata 4145			
Time	Concentration of Compound (µg/mL)		
(hours)	Acetylmexiletine	Acetyl-HMM	Acetyl-PHM
0	0.0	0.0	0.0
5	0.0	0.0	0.0
12	0.0	0.0	0.0
21	0.0	0.0	0.2
29	0.0	0.0	0.2
36	0.0	0.0	0.2
45	0.0	0.0	0.2
53	0.0	0.0	0.4
60	0.2	0.0	0.2
69	0.2	0.0	0.2
76	0.1	0.0	0.4
83	0.1	0.0	0.6
93	1.5	0.0	0.1
105	1.7	0.0	0.2
118	2.1	0.0	0.3
129	2.0	0.0	0.2
142	2.5	0.0	0.2
166	3.0	0.0	0.2
200	4.4	0.0	0.2

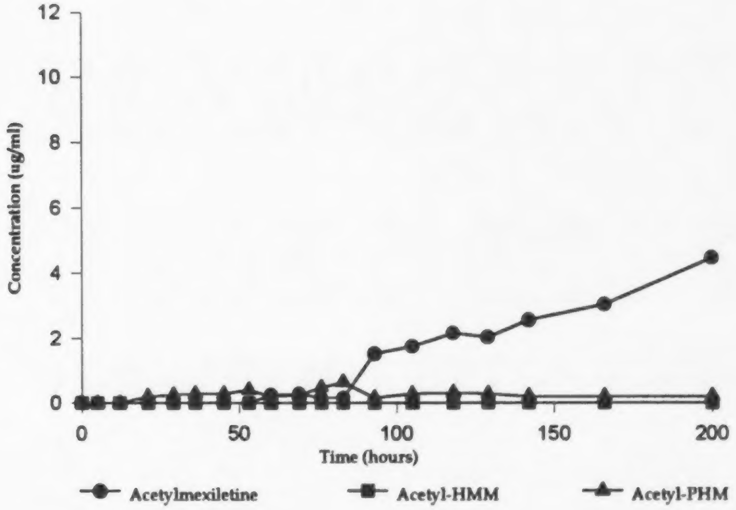


Figure 4- 34. A concentration time profile of acetylmexiletine, acetyl-HMM, and acetyl PHM biosynthesis in **czapec dox broth.**

Table 4-32. Analysis of **casamino acid broth** for the production of acetylated phase II metabolites from *rac*-mexiletine.

Cunninghamella echinulata 4145			
Time	Concentration of Compound (µg/mL)		
(hours)	Acetylmexiletine	Acetyl-HMM	Acetyl-PHM
0	0.0	0.0	0.0
5	0.0	0.0	0.0
12	0.0	0.0	0.4
21	0.0	0.0	0.2
29	0.0	0.0	0.2
36	0.0	0.0	0.2
45	0.0	0.0	0.0
53	0.0	0.0	0.2
60	0.0	0.0	0.2
69	0.0	0.0	0.2
76	0.0	0.0	0.2
83	0.0	0.0	0.4
93	0.0	0.0	0.5
105	0.0	0.0	0.8
118	0.0	0.0	0.8
129	0.4	0.0	1.4
142	1.6	0.0	0.8
166	2.0	0.0	0.7
200	2.0	0.0	0.8

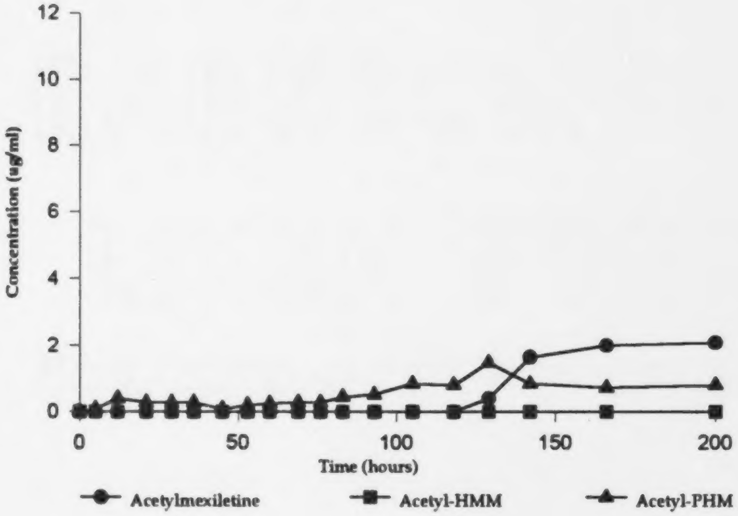


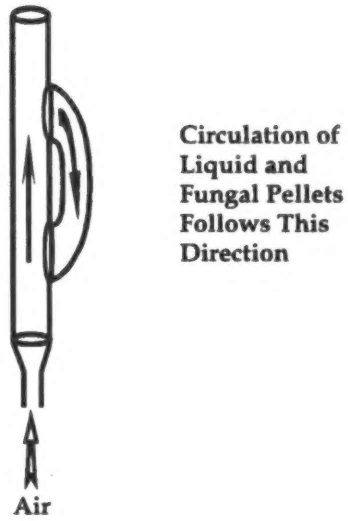
Figure 4-35. A concentration time profile of acetylmexiletine, acetyl-HMM, and acetyl PHM biosynthesis in **casamino acid broth**.

4.2.6 Scaling Up Biotransformation of *rac*-Mexiletine Using a D-Loop Fermentor and Yeast Extract Broth

When conditions for optimal metabolite biosynthesis were determined it was desirable to scale up the reaction using a D-Loop fermentor (figure 4-36). The bioreactor was filled with 500 mL of yeast extract medium at pH 7.0. In addition, a large quantity of rapidly growing mycelia was required to utilize this fermentor. It was prepared by propagating three phase I cultures in Erlenmeyer flasks (125 mL) using yeast extract medium. The biomass generated was used for an inoculum. The fermentor was then placed in a benchtop incubator set at 28 °C. A stock solution of *rac*-mexiletine was prepared, filter sterilized (22 µm pore size), and tested for its drug content using HPLC. The concentration of *rac*-

Figure 4-1. A representation of a D-Loop bioreactor that held 500 mL of liquid medium. A gas line was attached to the fermentor bottom and the subsequent air flow circulated the growth medium, and fungus, through the glass cylinder in a clockwise fashion (as depicted below).

D-Loop Bioreactor



mexiletine added to the D-Loop fermentor was determined to be 190.92 µg/mL. Results from this experiments are as follows:

Table 4-33. Metabolism of *rac*-mexiletine in 500 mL of yeast extract broth, at pH 7.0, in a D-Loop fermentor.

	Drug Enantiomer Concentration (µg/mL)					
Time	Mexiletine		HMM		PHM	
(hours)	S-(+)	R-(-)	S	R	S	R
0	95.5	95.5	0.0	0.0	0.0	0.0
1	39.4	35.2	0.0	0.0	0.0	0.0
10	29.3	22.4	0.0	0.0	0.0	0.0
22	29.1	21.8	0.0	0.2	1.5	3.2
29	33.6	27.0	0.8	0.6	2.4	5.4
36	27.0	18.1	1.0	0.8	2.4	6.4
45	21.4	12.8	0.7	0.5	0.2	5.3
60	20.0	12.7	1.0	0.7	2.3	6.7
70	16.7	7.2	1.2	0.8	2.6	7.2
78	36.0	20.4	2.0	1.6	3.3	11.2
85	31.2	21.0	2.4	2.0	4.4	12.7
94	39.6	19.5	2.5	2.0	3.7	13.0
102	40.4	21.3	2.6	2.5	3.8	14.8
117	48.0	21.5	3.3	2.6	4.7	15.6
124	35.2	23.8	2.6	2.0	4.8	14.4
131	34.3	20.0	2.6	2.0	3.6	13.0
142	39.4	22.0	3.2	2.4	5.6	16.3
153	36.6	26.5	3.1	3.0	4.8	16.0

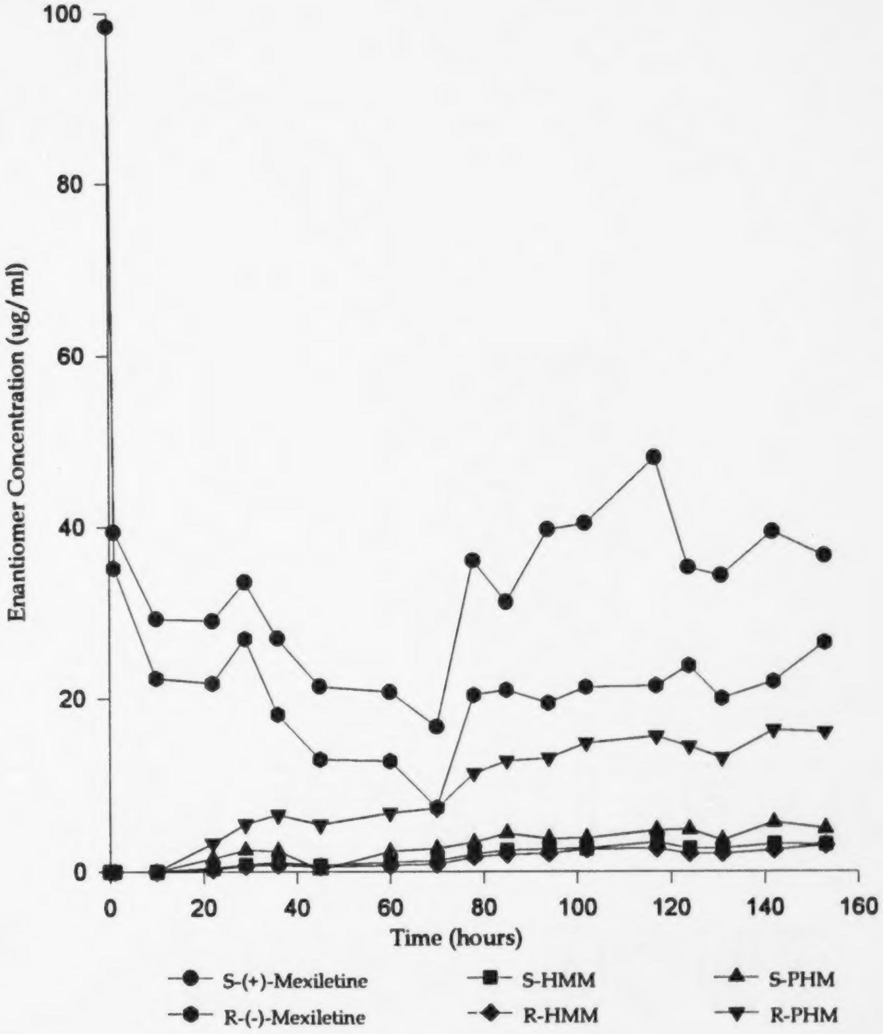


Figure 4-37. A concentration time profile of mexiletine, HMM, and PHM enantiomers in yeast extract broth from a D-Loop fermentor.

4.3 Discussion

Using *rac*-mexiletine as an *in vitro* substrate to study fungal models of mammalian metabolism revealed that *C. echinulata* 4145 was capable of stereoselectively biotransforming *rac*-mexiletine into the major human metabolites HMM and PHM. A summary of these results can be found in tables 4-36, 4-37, and 4-38. In general, the quantity of metabolite biosynthesized was strongly influenced by the nitrogen source utilized and medium pH. Furthermore, *C. echinulata* was capable of converting *rac*-mexiletine into the conjugated phase II metabolites, acetyl-mexiletine, acetyl-HMM and acetyl-PHM.

The first experiment conducted was designed to test the effect of various nitrogen sources on the ability of *C. echinulata* 4145 to metabolize *rac*-mexiletine. Overall, R-(-) and S-(+)-mexiletine enantiomers were not metabolized equally. R-(-)-mexiletine was preferentially removed from the growth medium with an average S-(+)/R-(-) ratio of 1.40 at the minimum concentration remaining[*]. In addition, R-PHM was preferentially biosynthesized with the average S/R ratio being 0.50 at the maximum concentration produced[**]. This preference for R-(-)-mexiletine correlates with human studies which indicated that this enantiomer preferentially attached to serum proteins and sodium channels, had greater antiarrhythmic activity, and was the favored glucuronide metabolite excreted by the kidney. However, this pattern was not observed with HMM biosynthesis where larger amounts of S-HMM were produced. The average S/R ratio was 1.13 at the maximum concentration produced. The identity of mexiletine diastereomers was confirmed by comparing run times to similarly derivatized R-(-)-mexiletine and S-(+)-mexiletine standards. Confirmation of the identity of HMM and PHM diastereomers was performed by comparing run times to similarly derivatized HMM and PHM biosynthesized by C. *echinulata* from R-(-)-mexiletine and S-(+)-mexiletine standards

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[*] Minimum amount of drug remaining over the 200 hour experiment. Values were chosen from semi-log graphs at the point where no decreases in drug concentration were observed.

[**] Maximum amount of metabolite biosynthesized over the 200 hour experiment. Values were chosen, from semi-log graphs, at the point where no increases in metabolite concentration were observed.

Data collected from the first experiment (table 4-1) indicated that the

Table 4-34. A summary of data obtained from the eight media preparations devised to test *rac*-mexiletine metabolism by C. *echinulata* 4145.

	Minimum Concentration of Mexiletine Remaining (μg/mL)							
	Yeast Extract	Trypticase Soy	Phosphate Buffer	Czapec Dox	Casamino Acids	Malt Extract	Peptone	Sabouraud Dextrose
S-(+)-mexiletine	14.9	13.4	26.5	43.6	44.6	45.2	22.3	16.2
R-(-)-mexiletine	7.6	10.4	23.6	36.2	29.8	46.0	16.1	9.4
Ratio of S-(+)-mex/ R-(-)-mex	1.97	1.29	1.12	1.21	1.50	1.00	1.39	1.71
Time required to reach minimum concentration (hours)	105	36	53	105	200	21	93	105

	Maximum Concentration of HMM Produced (μg/mL)							
	Yeast Extract	Trypticase Soy	Phosphate Buffer	Czapec Dox	Casamino Acids	Malt Extract	Peptone	Sabouraud Dextrose
S-HMM	17.8	12.7	8.1	10.9	9.5	4.9	12.05	11.9
R-HMM	12.3	11.8	7.7	10.3	7.7	5.2	9.85	11.8
Ratio of S-(+)-mex/ R-(-)-mex	1.44	1.10	1.05	1.06	1.23	0.93	1.22	1.00
Time required to reach minimum concentration (hours)	83	21	45	118	200	105	200	76

	Maximum Concentration of PHM Produced (μg/mL)							
	Yeast Extract	Trypticase Soy	Phosphate Buffer	Czapec Dox	Casamino Acids	Malt Extract	Peptone	Sabouraud Dextrose
S-PHM	24.1	15.3	10.2	11.9	14.3	6.9	16.4	12.5
R-PHM	40.5	32.2	20.4	25.7	34.0	10.5	39.2	26.6
Ratio of S-(+)-mex/ R-(-)-mex	0.61	0.48	0.50	0.46	0.42	0.65	0.42	0.47
Time required to reach minimum concentration (hours)	83	21	76	118	200	105	200	76

nitrogen source utilized had a dramatic effect on *C. echinulata*'s ability to metabolize *rac*-mexiletine. For example, when yeast extract broth was used, it proved to be the preferred nitrogen source for maximum HMM (S: 17.77 µg/mL, R: 12.31 µg/mL) and PHM (S: 24.06 µg/mL, R: 40.48 µg/mL) biosynthesis. Under these conditions 21% more HMM and 25% more PHM was detected when the results were compared to flasks employing trypticase soy broth. In addition, *C. echinulata* 4145 removed the largest concentration of *rac*-mexiletine [S-(+): 83.45 µg/mL, R-(-): 90.77 µg/mL] from flasks containing yeast extract broth. Finally, cultures containing yeast extract broth displayed the largest ratio between the enantiomers of mexiletine (S/R was 1.97) and HMM (S/R was 1.44). A difference was also observed in the ratio of PHM enantiomers biosynthesized (S/R was 0.61). Therefore, under these growth conditions, R-(-)-mexiletine was preferentially removed from the medium while R-PHM was preferentially biosynthesized. However, the opposite occurred with HMM production where S-HMM was produced in excess of R-HMM. This pattern was observed with all nitrogen sources tested.

A possible explanation for these results might be attributed to a component other than nitrogen (10%) contained within yeast extract broth. However, this would be difficult to prove because the manner in which yeast extract broth was produced made its exact composition difficult to define. Yeast extract broth is comprised of the water soluble material liberated during yeast cell autolysis. Therefore, it contains numerous preformed organic molecules specific for fungal growth (biotin, nicotinic acid). A future set of experiments could test the importance of a few of these in detail, using a minimal medium like czapec dox broth. The pattern of biosynthesis with czapec dox broth was responsible for the biosynthesis of 10.94 µg/mL S-HMM, 10.34 µg/mL R-HMM, 11.88 µg/mL S-PHM, and 20.42 µg/mL R-PHM. In total, this was 57% of the HMM and 60% of the PHM produced in yeast extract broth. By establishing baseline levels of metabolite production in a minimal medium, the effect of specific agents could be tested to determine whether they increased the concentration of HMM and PHM to levels seen in yeast extract broth.

Results obtained with czapec dox broth are mentioned at this point in the discussion to illustrate that C. *echinulata* 4145 can biosynthesize the metabolites HMM and PHM in a medium with a minimum number of components. The importance of this observation is contained within the described advantages of fungal models of mammalian metabolism. It was stated earlier that scaling up a biotransformation to yield semi-preparative amounts of metabolites was an important long term aspect of this work. However, when using a complex nitrogen source, there is the potential of co-purifying medium components along with compounds of interest. A minimal medium, containing the lowest number of components necessary for optimal metabolite biosynthesis, would decrease this risk. In addition, a defined medium would provide a backdrop from which drug metabolism reactions could be studied in more detail. If C. *echinulata*'s cytochrome P_{450} enzyme system could have been successfully isolated and quantified, metabolism, environmental conditions, and enzyme levels could have been examined and optimized in concert.

Results from another two nitrogen source experiments can be used to reveal the effect of medium components on metabolite biosynthesis. When trypticase soy broth for phase II growth was compared to phosphate buffer, it was observed that both environments yielded HMM and PHM. In addition, drug disappearance and metabolite production were stereoselective. However, there was 2.1 times more drug remaining (at minimum concentration remaining) in the trypticase soy broth medium than the phosphate buffer. There was also 1.55 times more PHM and 1.54 times HMM produced. An explanation for the observed differences can be formulated by comparing data obtained from phase II glucose utilization (Figure 3-9) with results obtained from biotransformation experiments using a medium containing yeast extract broth. Glucose utilization data indicated that the carbon source was completely consumed after 50 hours.

By superimposing this graph on figure 4-4 it would be observed that at 50 hours, the rate of drug disappearance and metabolite biosynthesis tapers off considerably. This provides evidence linking active growth with drug metabolism.

Even though sucrose utilization was not measured during growth in trypticase soy broth, a similar explanation holds. As was mentioned in section 3.4, fungal growth was difficult to quantify because fungi have the ability to store reserve substances. Consequently, increases in weight can occur without the formation of new hyphae. If a carbon source was not present, fungal growth could continue with the stored material. This explanation is consistent with observed results because the amount of drug consumed and metabolite produced is less in phosphate buffer than with trypticase soy broth. If growth was not a factor in biosynthesis, the metabolic profiles should have been identical. Therefore, fungal growth was an important requirement for optimal metabolite biosynthesis.

This observation emphasizes the need to develop a minimal medium for phase II growth. It would only contain components necessary for optimal metabolite biosynthesis. Therefore, it would decrease the risk of a medium component interfering with metabolite isolation and purification. In addition, if complex nitrogen sources, including yeast extract broth, trypticase soy broth, and peptone broth, were still required their use could be limited to generating phase I biomass. For phase II, a minimal medium that encouraged growth, could be incorporated to yield similar concentrations of metabolites. This would provide a simple, inexpensive modeling system to study drug metabolism *in vitro*.

Another two nitrogen sources providing high yields of HMM and PHM were casamino acids broth and peptone broth. When using casamino acid broth, *C. echinulata* 4145 biosynthesized 9.45 µg/mL S-HMM, 7.66 µg/mL R-HMM, 14.28 µg/mL S-PHM, and 34.01 µg/mL R-PHM. With peptone broth, 12.05

μg/mL S-HMM, 9.85 μg/mL R-HMM, 16.37 μg/mL S-PHM, and 39.24 μg/mL R-PHM was biosynthesized. In both cases S-HMM and R-PHM were the preferred enantiomers released back into the growth medium. A distinguishing feature of casamino acid broth and peptone broth use was that 200 hours (8.3 days) were required to reach maximum levels for metabolite biosynthesis. When compared to 83 hours (3.5 days) for yeast extract broth, 21 hours (0.9 days) for trypticase soy broth, 45 hours (1.9 days) for phosphate buffer and 118 hours (4.9 days) for czapec dox broth, it would appear that *C. echinulata* 4145 was active longer when casamino acid broth and peptone broth were employed. However, even though the amounts of metabolites produced were high, it would be more convenient to get similar results in a shorter period of time.

The nitrogen source experiment demonstrated biotransformation was stereoselective for R-(-)-mexiletine, R-PHM, and S-HMM. This data provided a basis from which a comparison could be made between the metabolism of *rac*-mexiletine and authentic S-(+) and R-(-) standards. A summary of results from such an experiment can be seen in table 4-35.

Table 4-35. A summary of data obtained from the metabolism of authentic S-(+) and R-(-) standards in yeast extract broth and trypticase soy broth.

	Trypticase Soy Broth			Yeast Extract Broth		
	Optimal conc. of remaining drug (μg/mL)	Time optimal concentration reached (hours)	% of drug added	Optimal conc. of remaining drug (μg/mL)	Time optimal concentration reached (hours)	% of drug added
	S-(+)-mexiletine Addition			S-(+)-mexiletine Addition		
S-(+)-mexiletine	0.0	20	0%	30.0	75	19%
S-HMM	0.1	75	0.06%	2.2	75	1.3%
S-PHM	33.4	75	21%	30.9	75	20%
	R-(-)-mexiletine Addition			R-(-)-mexiletine Addition		
R-(-)-mexiletine	0	30	0%	23.4	75	21%
R-HMM	1.0	69	0.7%	2.0	75	1.2%
R-PHM	82.4	60	51%	44.1	69	27%
Ratio of S-(+)-mex/R-(-)-mex	0			1.29		
Ratio of S-HMM/R-HMM	0.01			1.08		
Ratio of S-PHM/R-PHM	0.41			0.70		

When using trypticase soy broth, in conjunction with authentic S-(+) and R-(-) standards, the enantiomers were completely metabolized within 36 hours. This was different from results obtained with *rac*-mexiletine addition where there was detectable drug after 200 hours (concentrations of S-(+) and R-(-)-mexiletine were 11.41 μg/mL and 10.08 μg/mL respectively). In addition, while a low concentration of HMM was produced when the individual enantiomers were added, large increases in both S-PHM (2.2 times increase) and R-PHM (2.5 times increase) were detected. When yeast extract broth was used, a different pattern of metabolism was observed. A larger concentration of drug, than with racemate addition, remained in the medium at the final sampling time (75 hours). However, data were not collected for as long in this experiment. Also, as with trypticase soy broth, small amounts of HMM were produced in association with higher levels of PHM. The data would suggest that when pure enantiomers were used, aromatic hydroxylation was favored over benzylic.

A similarity between the addition of *rac*-mexiletine and the individual enantiomers was that R-(-)-mexiletine was the preferred enantiomer metabolized and R-PHM was the favored enantiomer biosynthesized. Therefore, as with human studies, the R-(-) antipode was preferentially oxidized by cytochrome P_{450}. However, an insufficient amount of data was obtained to determine whether there was a significant interaction between the enantiomers during metabolism.

This data indicates that the choice of nitrogen source significantly impacted metabolism. Therefore, a set of experiments was conducted to determine the effect of pH. When the nitrogen sources were initially tested, all media formulations were prepared in phosphate buffer at pH 7.0. We decided to continue this work by re-testing the nitrogen sources that yielded optimal metabolite at pH 5.0, 5.5, 6.0, 6.5, and 8.0. A summary of the data obtained can be found in table 4-36.

Table 4-36. A summary of data obtained from the metabolism of *rac*-mexiletine, by *C. echinulata* 4145, grown in yeast extract broth, trypticase soy broth, and peptone broth at pH 5.0, 5.5, 6.0, 6.5, and 8.0.

	Yeast Extract Broth							
	Initial Medium pH							
	5.5		6.0		6.5		8.0	
	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH
S-HMM	6.1	7.55	6.9	7.51	7.4	7.31	0	0
R-HMM	6.8	7.55	6.8	7.51	7.1	7.31	0	0
S-PHM	10.9	7.55	11.5	7.51	11.5	7.31	0	0
R-PHM	20.3	7.55	22.1	7.51	24.3	7.31	0	0

	Trypticase Soy Medium									
	Initial Medium pH									
	5.0		5.5		6.0		6.5		8.0	
	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH
S-HMM	7.9	8.33	8.5	8.16	7.5	8.37	10.5	8.16	0	0
R-HMM	8.2	8.33	7.4	8.16	6.3	8.37	9.5	8.16	0	0
S-PHM	11.5	8.33	12.8	8.16	12.4	8.37	12.3	8.16	4.7	7.92
R-PHM	22.4	8.33	25.0	8.16	24.5	8.37	25.4	8.16	10.3	8.19

	Peptone Broth							
	Initial Medium pH							
	5.5		6.0		6.5		8.0	
	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH	Max. conc. (µg/mL)	Medium pH
S-HMM	9.7	6.82	9.3	6.8	9.5	6.92	0	0
R-HMM	9.7	6.82	9.4	6.8	9.4	6.92	0	0
S-PHM	10.8	6.82	10.5	6.8	10.1	6.92	0	0
R-PHM	12.1	6.82	10.4	6.8	11.3	6.92	0	0

When the effect of pH was tested using yeast extract broth, metabolite biosynthesis increased with pH (5.5 to 6.5). However, at pH 8.0 metabolites were not detected. At pH 5.5, 12.88 µg/mL of R+S-HMM, and 31.35 µg/mL of R,S-PHM were produced compared to 14.48 µg/mL of R+S-HMM and 35.78 µg/mL of R,S-PHM at pH 6.5. This corresponded to a 13% increase in HMM and 15% increase in PHM biosynthesis. Data obtained from *C. echinulata* 4145 grown in yeast extract broth (table 4-1) at pH 7.0 indicated this combination generated the largest concentration of metabolites. Therefore, the combination of these two results would indicate that pH 7.0 or 7.5 was optimal for biosynthesis.

This study indicated changes in pH did not effect the stereoselective nature of metabolite biosynthesis. For PHM production the S/R ratio was stable over the pH range tested (at pH 5.5, S/R was 0.54; at pH 6.5, S/R was 0.49). However, a subtle change was detected with HMM production in all cultures tested. As pH increased during incubation, a preference for R-HMM production was changed into one for S-HMM (with yeast extract broth, ratio increased from 0.89 to 1.04). In order to verify this change and determine whether it was significant, more data would have to be collected.

Another trend observed with cultures grown in yeast extract broth was that the pH at optimal metabolite concentration was similar in all incubates tested. The measured values were 7.55, 7.51, and 7.31 even though their starting points were 5.5, 6.0, and 6.5. In addition, increases in pH beyond 7.55 caused a decrease in metabolite concentration.

Graphical representations of these data (figures 4-21, 4-22), linking pH with metabolite biosynthesis, are a useful way to observe the effect of medium pH on metabolite biosynthesis directly. With the three acidic yeast extract broth preparations (pH 5.5, 6.0, 6.5), a decrease in medium pH was observed over the first 15 hours followed by a gradual increase to pH 8.0. The initial decrease (acidification) can be attributed to acid by-products released into the medium during carbohydrate metabolism. The subsequent increase in pH can be linked to utilization of buffer components for cell growth. In addition, an observed trend was that an increase in pH correlated with metabolite biosynthesis. More specifically, metabolites were not produced until the pH increased.

If it is true that a pH above 7.6 was associated with the cessation of metabolite production in yeast extract broth, the biosynthesis of HMM and PHM could be sustained by increasing the media's buffering capacity. Another solution would be to control the pH by the addition of diluted acid throughout the incubation.

Problems associated with a basic pH were also observed when trypticase soy broth was used. In general, as pH increased from 5.0 to 6.5, the concentration of metabolites increased from 16.12 μg/mL R+S-HMM and 33.89 μg/mL R+S-PHM to 18.00 μg/mL R+S-HMM and 37.68 μg/mL R+S-PHM. This corresponded to a 12% increase for HMM and an 11% increase in PHM biosynthesis. In addition, at an initial medium pH of 8.0 there was 16.11 μg/mL PHM biosynthesized but no HMM. This data was in agreement with the previous finding that *C. echinulata* 4145 prefers a pH around 7.0 for optimal metabolite production. In addition, appearance of metabolite was preceded by an increase in pH. This includes PHM produced in trypticase soy broth at an initial pH of 8.0.

When peptone broth was used as the nitrogen source, HMM and PHM concentration decreased as the medium pH increased from 5.5 to 6.5. Furthermore, there were no metabolites detected at pH 8.0. In this study, data were collected for a total of 60 hours making it difficult to compare numbers with results obtained in the original study (200 hours). When yeast extract broth and trypticase soy broth were utilized, the medium pH would turn basic during the incubation. This increase in pH was correlated with the dissappearance of biosynthesized metabolite from the medium. With peptone broth, this was not observed. However, appearance of metabolite was preceded by an increase in pH.

When malt extract broth and Sabouraud dextrose broth were initially used as the nitrogen source, they were adjusted to pH 7.0. However, the characteristic pH for these media formulations was 4.7 and 5.7 respectively. This difference in pH had a significant impact on metabolite biosynthesis. Using malt extract broth at pH 7.0, 10.09 μg/mL R+S-HMM and 17.36 μg/mL R+S-PHM were produced compared to non-detectable concentrations at pH 4.7. When Sabouraud dextrose broth was used at pH 7.0, 23.56 μg/mL R+S-HMM and 39.09 μg/mL R+S-PHM was biosynthesized compared to 9.05 μg/mL R+S-HMM and 11.18 μg/mL R+S-PHM at pH 5.7 (table 4-37). This corresponds to a 62% decrease in HMM and a 71% decrease in PHM biosynthesis. Therefore, these data support the observation that *C. echinulata* prefers a neutral pH for optimal metabolite production. In addition, the appearance of metabolite in Sabouraud dextrose broth was preceded by an increase in pH. Therefore, future experiments could determine the significance of increases in pH, in addition to developing a system preventing the pH to increase above 7.8.

Table 4-37. A summary of data obtained from the metabolism of *rac*-mexiletine, by *C. echinulata* 4145, grown in Sabouraud dextrose broth at 5.7.

	Sabouraud Dextrose Broth (pH 5.7)	
	Max. conc. (μg/mL)	Medium pH
S-HMM	4.7	7.65
R-HMM	4.3	7.65
S-PHM	0.0	N/M
R-PHM	11.2	7.65

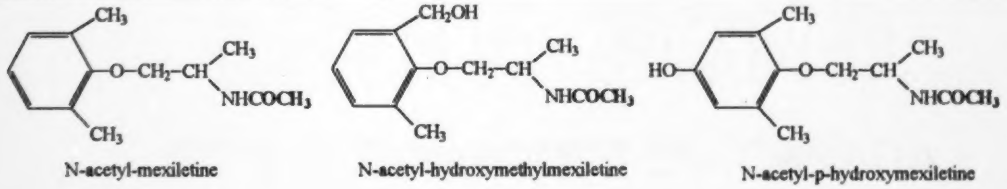
N/M - Not measured.

A final observation regarding the changes in medium pH was that it had a consistent effect on fungal growth for all nitrogen sources tested. As pH was increased from 5.5 to 8.0, fungal morphology changed from small, dense individual pellets (pH 5.5) to diffuse mycelia (pH 8.0) growing without an overall form (appearance was reminiscent of submerged tissue paper). This change in appearance occurred in small increments over the pH range tested with the individual pellets slowly congealing until a single mass of widely separated mycelia was visible. Therefore, since cultural morphology was pH sensitive, it had the potential of being calibrated for optimal metabolite biosynthesis. The form of growth occurring at pH 7.5 could be used as an indicator for optimal metabolite biosynthesis.

When bioconversion of *rac*-mexiletine was examined in more detail, the average recovery of drug and metabolite was calculated to be between 45% and

70% depending on the nitrogen source utilized. Therefore, a significant concentration of drug was unaccounted for. A possible location for these products was the fungal cytoplasm[*]. However, when this possibility was evaluated using HPLC, the maximum amount of drug detected was 6.7 µg/mL of S-(+), R-(-)-mexiletine and 0.9 µg/mL R+S-PHM. The remaining drug was either incorporated into cellular material or eliminated as another metabolite(s). Our analytical procedure was only capable of detecting basic drugs possessing a primary or secondary amino group. The possibility existed that a neutral or acidic metabolite was being biosynthesized.

Reports by Smith and Rosazza (1974) and Coutts *et al.* (1979) indicated that fungi were capable of acetylating the amino group of aniline and *rac*-N-alkyl-amphetamines. Therefore, it was possible *rac*-mexiletine was being partially eliminated as an acetylated metabolite because it possessed a similar amino group and is structurally similar to amphetamines. To investigate the possible formation of these phase II metabolites, an HPLC method was developed to re-test broth samples collected from the nitrogen source determinations. However, the analytical method was designed to detect the neutral metabolites AMEX, AHMM, and APHM (figure 4-38). Authentic



N-acetyl-mexiletine   N-acetyl-hydroxymethylmexiletine   N-acetyl-p-hydroxymexiletine

Figure 4-38. Structure of the N-acetyl metabolites, AMEX, AHMM, and APHM, formed during the biotransformation of *rac*-mexiletine.

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[*] Testing for intracellular drug and metabolite was accomplished by examining a 1.0 mL sample, of the mycelia obtained from a 125 mL Erlenmeyer flask, after being ground with a homogenizer.

standards were synthesized in our laboratory and their structures were confirmed using mass spectral analysis. Identification of the acetylated drug and metabolites was confirmed by comparing run times to acetylated standards.

The maximum concentration (14.8 μg/mL total metabolites) of AMEX, AHMM, and APHM was obtained using trypticase soy broth (table 4-38). This corresponded to the recovery of an additional 7.0% of added drug. The least amount was recovered in yeast extract broth accounting for 1.8% of added drug. Even though the amount of metabolite recovered was small, it emphasized that *C. echinulata* 4145 possessed additional mechanisms capable of eliminating a foreign compound.

Table 4-38. A summary of the concentrations of phase II acetylated metabolites obtained from the metabolism of *rac*-mexiletine by *C. echinulata* 4145 using various nitrogen sources.

	Maximum Biosynthesis of Acetylated Metabolites (μg/mL)					
	Trypticase Soy Broth	Phosphate Buffer	Yeast Extract Broth	Peptone Broth	Czapek Dox Broth	Casamino Acid Broth
Acetyl-mexiletine	4.3	4.4	0.5	5.2	4.5	2.0
Acetyl-HMM	2.2	0	0.8	0.8	0	0
Acetyl-PHM	8.3	3.4	2.0	2.5	0.6	1.5
Total Metabolite	14.8	7.8	3.3	8.5	5.1	3.5

N-oxidation is another mechanism used by *C. echinulata* to metabolize drugs with primary or secondary amino groups. This route of elimination was reported by Coutts *et al.* (1979) after they determined *C. echinulata* ATCC 9244 was capable of biosynthesizing a hydroxylamine from the substrate N-(n-propyl)amphetamine. In addition, a hydroxylamine was one of the reported mammalian metabolites for *rac*-mexiletine metabolism. Therefore, the possibility existed that a portion of added *rac*-mexiletine was being converted into this metabolite. However, we did not try to identify this, or acidic metabolites, from the fermentation medium.

The final experiment conducted was an attempt to scale-up the production of HMM and PHM biosynthesis using a D-Loop fermentor. The conditions chosen to accomplish this were based on previous results which suggested that yeast extract broth at pH 7.0 would provide optimal results. However, we found the concentration of metabolites biosynthesized in the D-Loop fermentor (500 mL) to be much less than the amount detected in Erlenmeyer flasks (125 mL). When the 500 mL bioreactor was operated for 142 hours, 39.44 μg/mL S-(+)-mexiletine and 22.00 μg/mL R-(-)-mexiletine was detected. This was 32% of the drug added or 2.5 times the concentration of *rac*-mexiletine remaining in 125 mL Erlenmeyer flasks under the same conditions. In addition, 3.2 μg/mL S-HMM, 2.48 μg/mL R-HMM, 5.6 μg/mL S-PHM, and 16.32 μg/mL R-PHM were biosynthesized after 142 hours. This corresponded to a 3.0% yield of S,R-HMM and an 8.5% yield of S,R-PHM. Furthermore, this was an 81% decrease in HMM and a 66% decrease in PHM biosynthesis from the Erlenmeyer flasks.

An explanation for these poor results can be attributed to the way *C. echinulata* 4145 grew in the D-Loop fermentor (Figure 4-39). An inoculum of 25.63 g (wet weight) was utilized for this experiment, which in combination with the rich growth medium, providing considerable growth in a matter of hours.

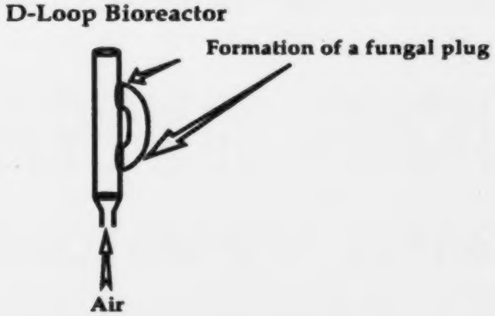


Figure 4-39. A diagrammatic representation of the location of fungal plugs formed during a biotransformation experiment.

This would have effected the pH, decreasing metabolite biosynthesis. In addition, fungal growth occurred so quickly that it plugged the fermentor preventing circulation of the medium. Therefore, the same effort required to optimize fungal growth in Erlenmeyer flasks (see chapter 3) would be required to determine optimal conditions for metabolite biosynthesis. A better solution might be to avoid D-Loop bioreactors and use larger Erlenmeyers.

4.4 Conclusions

Using *rac*-mexiletine as an *in vitro* substrate to study fungal models of mammalian metabolism indicated that *C. echinulata* 4145 was capable of stereoselectively biotransforming *rac*-mexiletine into the major human metabolites HMM and PHM. In addition, it has been reported that the isozyme responsible for the production of these metabolites in humans was CYP2D6. Experiments conducted by Foster *et al.* (1989), demonstrated that sparteine and quinidine decreased the rate and extent of methoxyphenamine biotransformation by the same fungus, *C. echinulata* 4145. Therefore, since *rac*-mexiletine and methoxyphenamine are substrates for the same enzymes in humans, the enzyme responsible for HMM and PHM biosynthesis in our studies may be similar to CYP2D6.

In general, the quantity of metabolite biosynthesized was strongly influenced by the nitrogen source utilized and medium pH. Furthermore, *C. echinulata* was capable of converting *rac*-mexiletine into the conjugated, phase II metabolites, acetyl-mexiletine, acetyl-HMM and acetyl-PHM.

5.0 Fungal Biotransformation of *rac*-Propranolol

5.1 Introduction

rac-Propranolol is a nonselective β-adrenergic blocking agent used in the management of hypertension, angina pectoris, and certain kinds of cardiac arrhythmias (Frishman and Charlap 1988). It functions by competitively blocking β-adrenergic receptors within the myocardium, bronchial smooth muscle, and vascular smooth muscle thereby inhibiting the response to adrenergic stimuli (Harvey *et al.* 1992). Clinically, propranolol is used as a racemic mixture of the R (rectus) and S (sinister) isomers. However, it has been demonstrated that S-propranolol is approximately 100 times more potent as a β-blocking agent than the corresponding R isomer in humans (Barret and Cullum 1968). In addition, upon administration of the racemic mixture, R-(+) and S-(-)-propranolol have varied kinetics including differences in plasma protein binding, metabolism, and elimination (Walle *et al.* 1988). For example, studies examining the clearance (oral administration) of *rac*-propranolol in humans revealed that even though interindividual variability was large, plasma concentrations of S-(-)-propranolol exceeded those of R-(+)-propranolol by as much as 90% (Prakash *et al.* 1989).

Upon oral administration, *rac*-propranolol was almost completely absorbed with peak concentrations occurring within 90 minutes (Patterson *et al.* 1970). It distributes widely throughout the body and becomes highly bound (85 to 96%) to plasma proteins (Borga *et al.* 1977). Elimination occurs almost exclusively by metabolic transformation in the liver followed by renal excretion of the products. Approximately 0.15 to 0.70% of an oral dose can be recovered as unchanged drug in urine (Walle *et al.* 1985). In addition, an oxidative metabolite, 4-hydroxypropranolol (4HOP), possessed β-adrenergic blocking activity equipotent to *rac*-propranolol (Fitzgerald and O'Donnell 1971). As a consequence, the metabolism of this drug has a direct impact on therapy.

When the human metabolism of *rac*-propranolol was examined in greater detail, it was observed that the majority of products arose from three initial reactions (Figure 5-1). They were direct glucuronidation of the parent drug (17%), ring oxidation (42%) to form 4HOP (86% of ring oxidation), 5HOP (11% of ring oxidation), plus 7HOP (<3% of ring oxidation), and side chain oxidation (41%) to form N-desisopropyl propranolol (DIP) [Walle *et al.* 1985]. The predominant hydroxy metabolite, 4HOP, was mainly recovered as two conjugates identified as 4-hydroxypropranolol glucuronide (4HOPG) and 4-hydroxypropranolol sulfate (4HOPS). Furthermore, DIP was metabolized by monoamine oxidase to an intermediate aldehyde, which was either further oxidized to naphthoxylactic acid (NLA) or reduced to propranolol glycol (Chen and Nelson 1982). Side chain oxidation accounted for approximately 41%, of an administered dose, half of which was recovered in urine as NLA (Figure 5-1). In addition, the R-(+) enantiomer was eliminated more efficiently than the biologically active S-(-) enantiomer, due in part, to the stereoselectively of the individual pathways involved (Marathe *et al.* 1994). To illustrate, Walle and Walle (1991) demonstrated that at low *rac*-4HOP concentrations, phenolsulfotransferase had a 4.6 fold greater affinity for the R-(+) enantiomer. However, the difference decreased as 4HOP concentration increased. It is important to emphasize that there are species differences in the way *rac*-propranolol is processed. For example, the S-(-) enantiomer was eliminated more efficiently than its R-(+) antipode in rats and dogs (Von Bahr *et al.* 1982). Therefore, one must be careful when comparing data obtained from various models, to human studies.

As with *rac*-mexiletine, it was established that hydroxylation of *rac*-propranolol was mediated by the polymorphic CYP2D6 (Ward *et al* 1989). However, 4HOP was still biosynthesized in significant amounts in PMs of debrisoquine, suggesting the involvement of P_{450}s other than CYP2D6. This

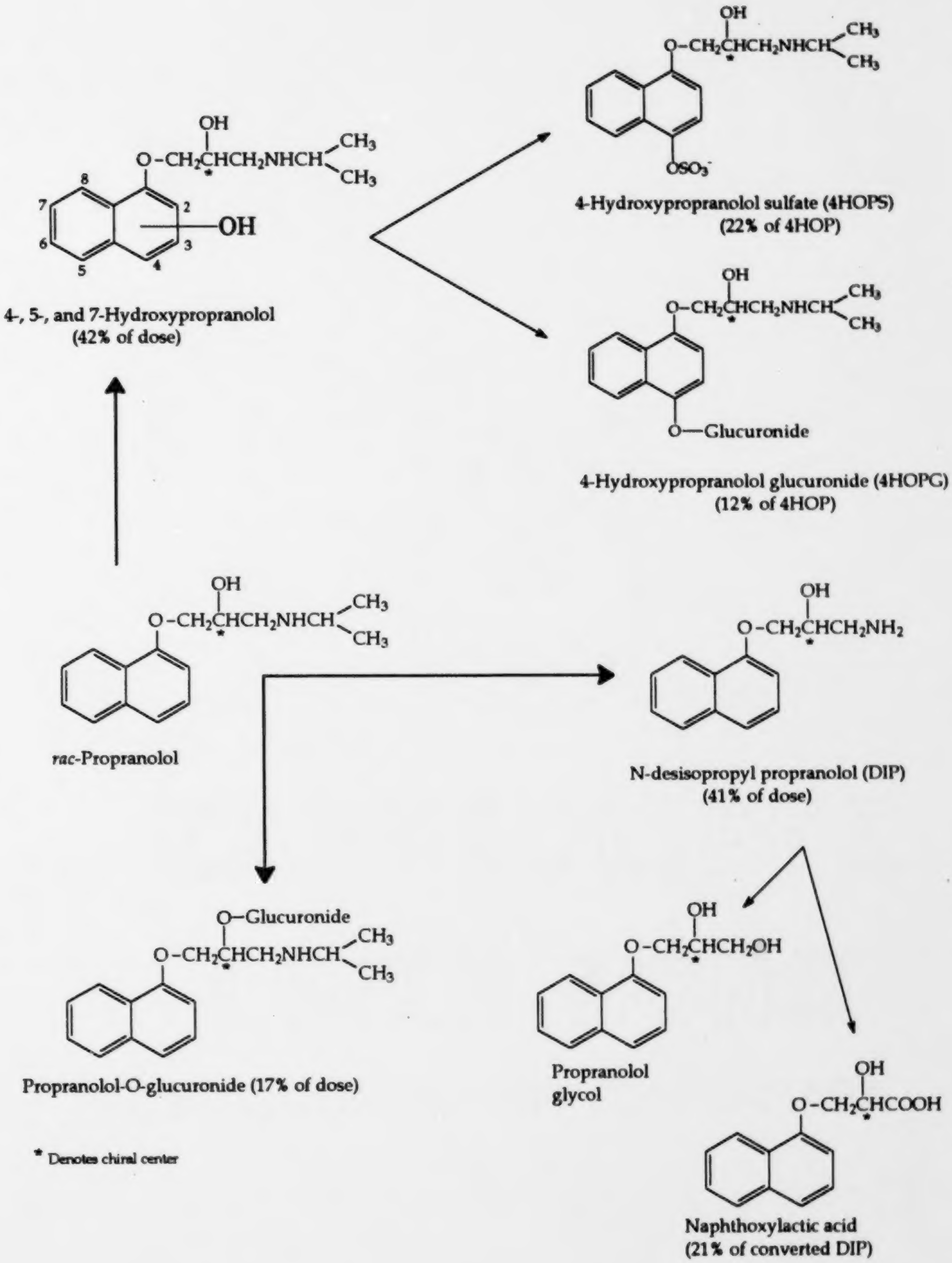

OH
O-CH2CHCH2NHCH
CH3
CH3
8
7
6
5
4
3
2
OH
4-, 5-, and 7-Hydroxypropranolol
(42% of dose)
OSO3-
4-Hydroxypropranolol sulfate (4HOPS)
(22% of 4HOP)
O—Glucuronide
4-Hydroxypropranolol glucuronide (4HOPG)
(12% of 4HOP)
rac-Propranolol
O-CH2CHCH2NH2
N-desisopropyl propranolol (DIP)
(41% of dose)
O—Glucuronide
Propranolol-O-glucuronide (17% of dose)
* Denotes chiral center
O-CH2CHCH2OH
Propranolol glycol
O-CH2CHCOOH
Naphthoxylactic acid
(21% of converted DIP)

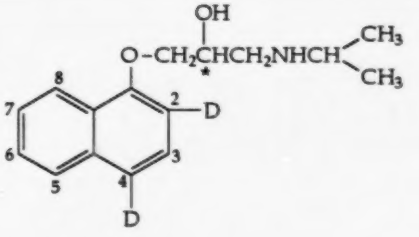
Figure 5-1. A summary of the principal routes of *rac*-propranolol metabolism in humans as well as significant secondary products.

study also determined that patients who were PMs of mephenytoin, a specific substrate for CYP2C (Smith 1991), formed less NLA than EMs.

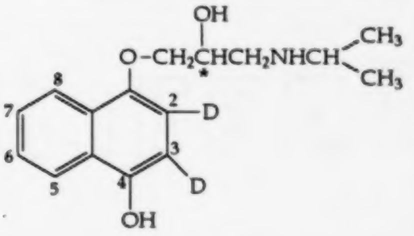
Metabolism of *rac*-propranolol was also studied using human liver microsomes (Marathe *et al* 1994). Enantioselective formation of the hydroxy metabolites and DIP were characterized using deuterium labeled (position 3 in propanolamine chain) pseudoracemic propranolol (S-(-) was only enantiomer labeled). Results indicated that at least two enzymes were involved in the formation of each of these two metabolites. In addition, R-(+)-propranolol was preferentially metabolized into 4HOP and 5HOP. Preference for the R-(+) enantiomer was also observed with DIP formation. Another aim of the study was to characterize the specific contribution of CYP2D6 using quinidine as an inhibitor. From this data it was observed that quinidine decreases 4HOP and 5HOP production whereas N-dealkylation was unaffected. Therefore, CYP2D6 was partly responsible for the appearance of these metabolites.

Overall, the preceeding studies demonstrated that *rac*-propranolol was extensively metabolized after administration to humans. Approximately 82% of an oral dose was stereoselectively oxidized by cytochrome P_{450}. In addition, the oxidative profile was found to be species dependent. From these observations, *rac*-propranolol would be an excellent probe for studying mammalian metabolism *in vitro*. As was previously discussed, the metabolism of *rac*-propranolol has been studied using human liver microsomes. Another *in vitro* model used to study the oxidative biotransformation of *rac*-propranolol was *Cunninghamella bainieri*. Foster *et al.* (1989b), examined whether this fungus could generate a metabolic profile similar to the one found in humans. In that study, using a substrate concentration of 120 μg/mL, they were able to detect five oxidative metabolites. These included 4HOP (0.22μg/mL), DIP (0.005 μg/mL), propranolol glycol (0.01 μg/mL), and trace amounts of NLA and 1-naphthoxy acetic acid; these correspond to the metabolites reported in humans. Broth samples were also treated with the enzyme ketodase to determine whether conjugated metabolites were biosynthesized. After reacting aliquots of fermentation medium with this enzyme, it was found to contain 0.822 µg/mL of 4HOP. The exact identity of this metabolite, derived from enzymatic hydrolysis of the 4HOP conjugate, was not determined. In addition, metabolite biosynthesis was inhibited by co-administering quinidine or sparteine, suggesting the involvement of a CYP2D6-like enzyme (Foster *et al.* 1989b). In a subsequent study, Foster *et al.* (1992), reported the formation of a new hydroxy metabolite whose structure was determined to be 8-hydroxypropranolol. In addition, 4HOP was detected in trace amounts. They suggested that a new batch of trypticase soy media was responsible for the decrease in production. This was consistent with the present report where fermentation medium had a significant effect on metabolism.

Experiments conducted by Foster *et al.* (1989b, 1992) indicated that fungal models were a useful tool for generating relevant metabolic data *in vitro*. Based on their findings it was possible to improve the yield of 4HOP, an active metabolite equipotent to propranolol, using information obtained with our metabolic study of *rac*-mexiletine. In addition, by labeling *rac*, R-(+), and S-(-)-propranolol with deuterium at positions 2 and 4 of the naphthyl ring (Chapter 2.7), it was possible to biosynthesize *rac*, R-(+), and S-(-)-4HOP with a stable



Deuterium labeled *rac*-propranolol



Deuterium labeled *rac*-4HOP

* Denotes chiral center

Figure 5-2. Structures of deuterium labeled *rac*-propranolol and the corresponding deuterium labeled metabolite, deuterated 4HOP.

isotope. It has been reported that C. *echinulata* incorporates molecular oxygen using the NIH shift (Auret *et al.* 1971). Therefore, the 4HOP produced will have deuterium labels at positions 2 and 3 on the naphthyl ring (Figure 5-2).

5.2 Results

5.2.1 Effect of Nitrogen Source

As was discussed in chapters 3 and 4, fungal growth and metabolite biosynthesis was affected by environmental conditions and the nitrogen source employed. Therefore, an experiment was designed to test the influence of three nitrogen sources on the biotransformation of *rac*-propranolol (Table 5-1 and Figures 5-4 to 5-6).

Biotransformations were achieved in 125 mL Erlenmeyer flasks, grown at 28°C, in an environmental shaker. A stock solution of *rac*-propranolol was prepared (50 mg/mL), filtered sterilized (0.22 μm pore size) and 100 μl portions were added to each flask. The concentrations of *rac*-propranolol and *rac*-4HOP in the fermentation broth were determined using the HPLC method described in chapter 2.5.3. However, due to the appearance of an unknown metabolite (Figure 5-3) that interfered with R-4HOP and S-4HOP quantitation, only the concentrations of R-(+) and S-(-) propranolol are listed.

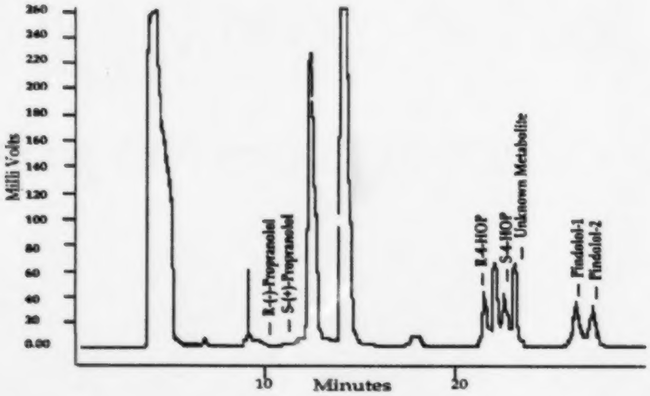


Figure 5-3. Chromatogram of 18 hour yeast extract broth that was inoculated with 300 μg/mL *rac*-propranolol.

Table 5-1. Concentration profile obtained by examining the metabolism of *rac*-propranolol at pH 7.0 using the nitrogen sources indicated.

	Yeast Extract Broth		Peptone Broth		Trypticase Soy Broth	
Time (hours)	R-(-)-Propranolol (μg/mL)	S-(+)-Propranolol (μg/mL)	R-(-)-Propranolol (μg/mL)	S-(+)-Propranolol (μg/mL)	R-(-)-Propranolol (μg/mL)	S-(+)-Propranolol (μg/mL)
0	100.0	100.0	100.0	100.0	100.0	100.0
1	56.6	57.9	68.9	68.2	46.4	45.6
3	51.1	51.3	47.1	45.1	50.8	48.2
5	45.5	45.8	44.1	40.1	45.7	42.4
7	39.8	39.8	30.2	28.3	44.4	33.5
9	35.5	35.7	18.8	17.4	26.8	28.3
11	32.9	33.1	12.9	11.6	21.6	18.6
13	28.5	28.5	11.9	10.5	10.0	8.0
15	23.1	23.4	10.1	9.0	0.7	0.0
22	14.7	14.2	3.4	2.8	0.0	0.0
24	13.6	13.6	2.1	0.0	0.0	0.0
26	11.3	10.5	0.9	0.0	0.0	0.0
30	8.6	8.0	0.0	0.0	0.0	0.0
33	6.2	5.7	0.0	0.0	0.0	0.0
35	4.8	4.3	0.0	0.0	0.0	0.0
37	4.1	3.6	0.0	0.0	0.0	0.0
46	2.1	1.6	0.0	0.0	0.0	0.0
53	0.0	0.0	0.0	0.0	0.0	0.0
70	0.0	0.0	0.0	0.0	0.0	0.0

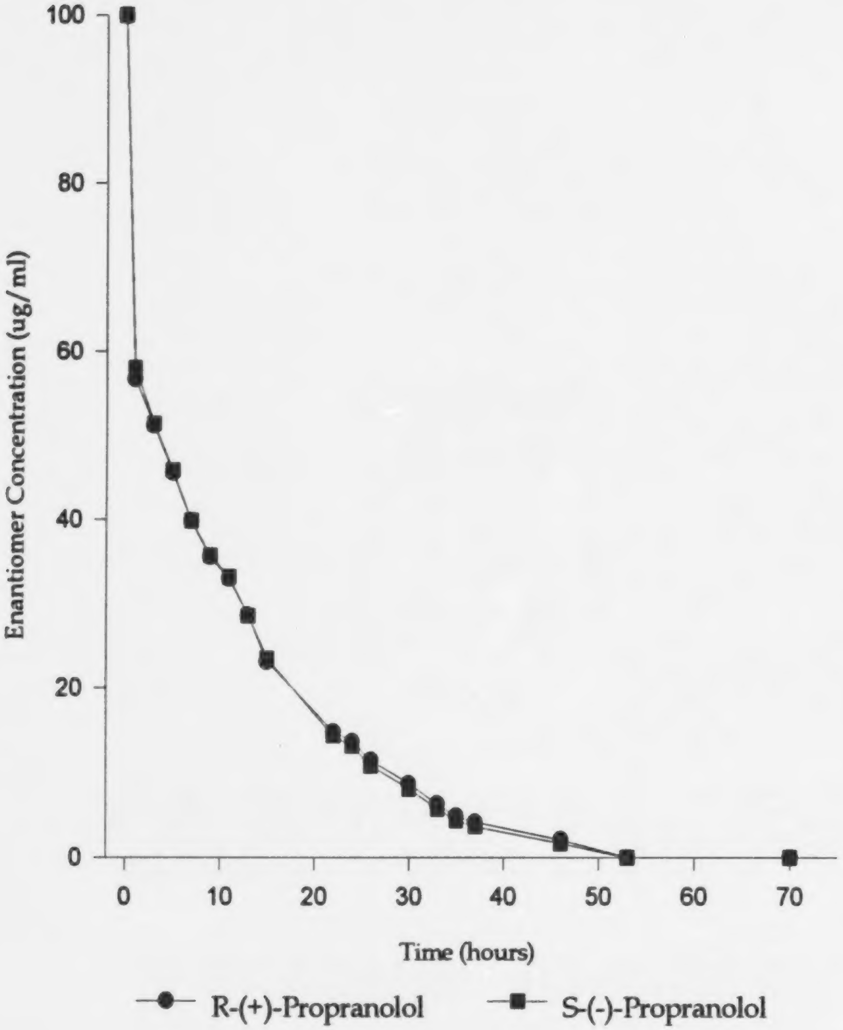


Figure 5-4. A concentration time profile of propranolol enantiomers in yeast extract broth (pH 7.0).

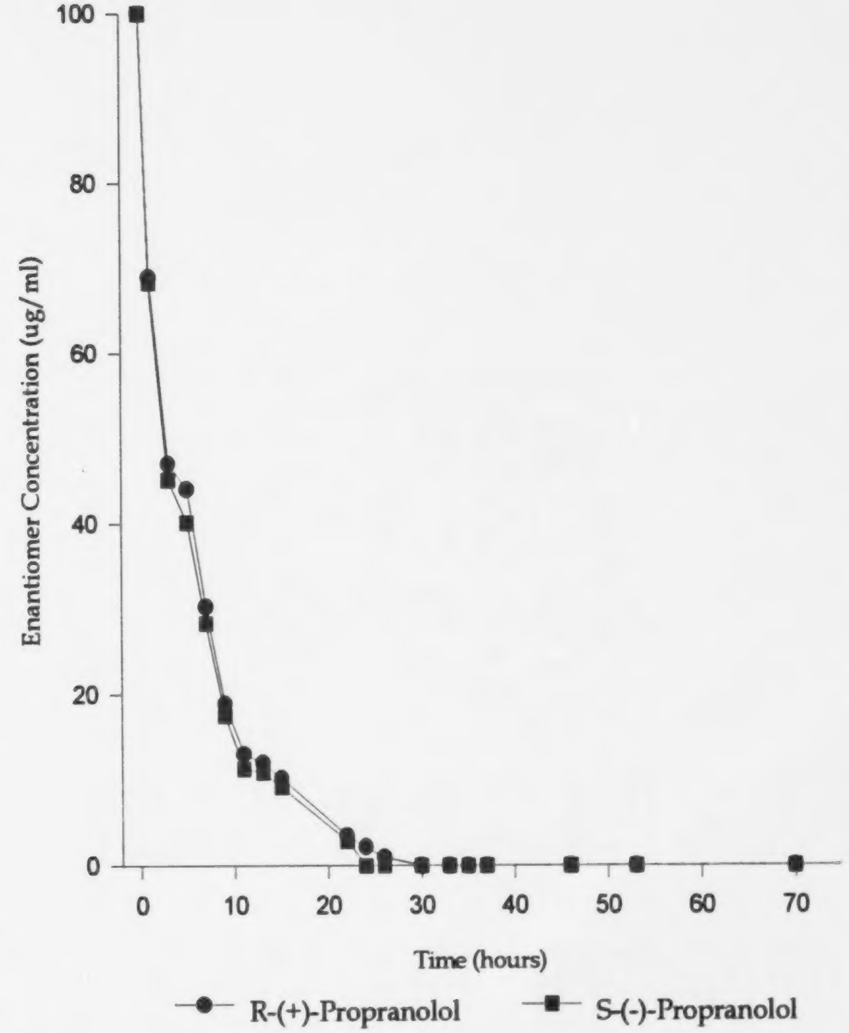


Figure 5-5. A concentration time profile of propranolol enantiomers in **peptone broth** (pH 7.0).

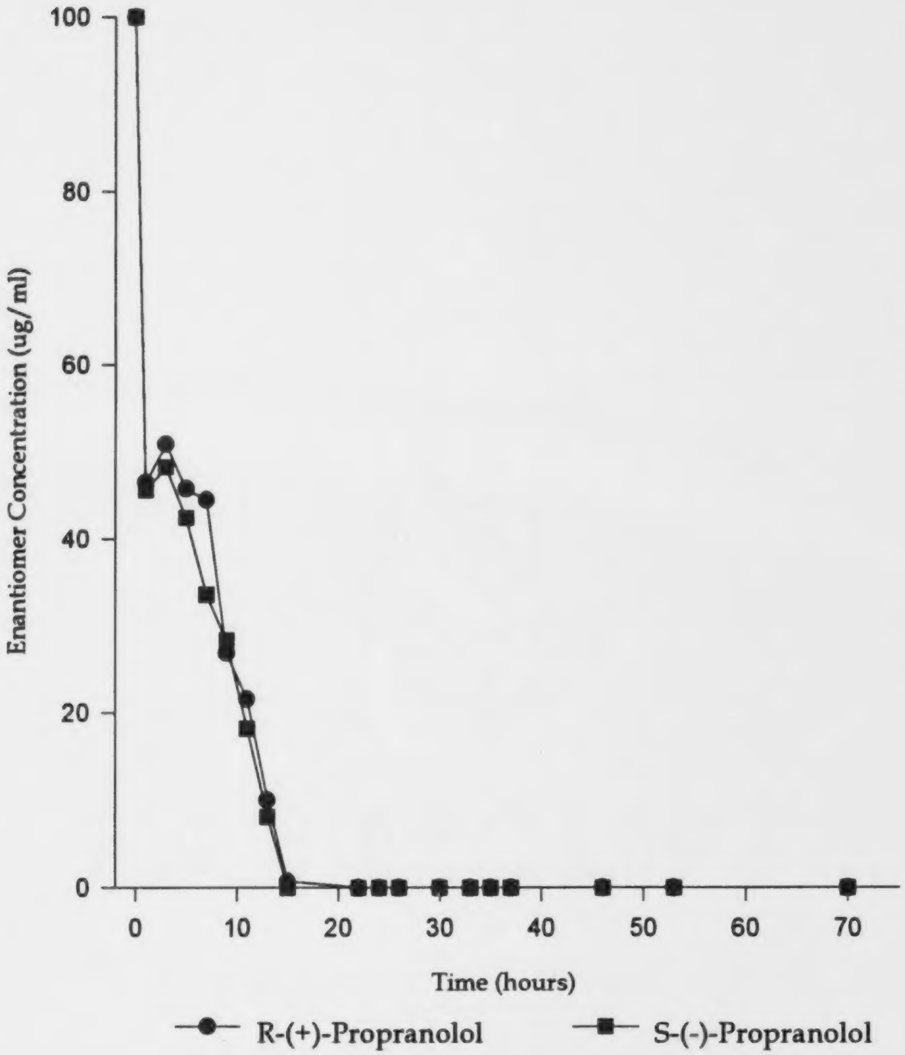
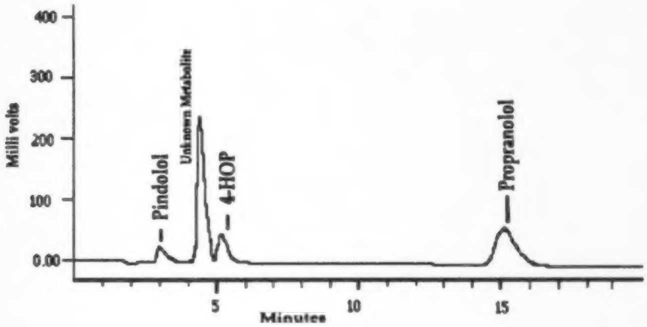


Figure 5-6. A concentration time profile of propranolol enantiomers in trypticase soy broth (pH 7.0).

5.2.2 Testing the Effect of Substrate Concentration on 4-Hydroxypropranolol Biosynthesis.

As we were unable to quantify the amount of 4HOP biosynthesized by *C. echinulata* 4145, using a stereospecific HPLC assay, a non-stereospecific procedure was employed (Chapter 2.5.4). Our rationale was that with fewer peaks in a non-stereospecific assay, the unknown metabolite would be less likely to interfere with 4HOP quantification. As can be seen in the chromatogram below, this unknown metabolite was still detected with the reverse phase assay developed. However, its retention time was sufficiently different from 4HOP such that it did not interfere with our ability to quantify 4HOP.



With this assay, a series of experiments was designed to determine the optimal conditions for 4HOP and deuterated 4HOP biosynthesis. The first experiment was conducted to determine whether the concentration of *rac*-propranolol, added to the fermentation medium, influenced 4HOP biosynthesis (Table 5-2 and Figures 5-7 to 5-9). Yeast extract was chosen as the growth medium for this experiment because it gave optimal results with *rac*-mexiletine. Stock solutions of S-(-), R-(+), and *rac*-propranolol and deuterated S-(-), R-(+), and *rac*-propranolol were prepared, filter sterilized (0.22 μm pore size), and tested for their exact drug content using HPLC. This determination was found to be necessary because the drug was adsorbing onto the filter matrix.

Table 5-2. Concentration of propranolol, and 4HOP, detected in **yeast extract broth** following addition of the varying amounts of substrate indicated below.

	Effect of adding 100 µg/mL *rac*-propranolol on 4HOP biosynthesis.		Effect of adding 200 µg/mL *rac*-propranolol on 4HOP biosynthesis.		Effect of adding 400 µg/mL *rac*-propranolol on 4HOP biosynthesis.	
Time (hours)	Propranolol (µg/mL)	4HOP (µg/mL)	Propranolol (µg/mL)	4HOP (µg/mL)	Propranolol (µg/mL)	4HOP (µg/mL)
0	90.4	0.0	179.4	0.0	358.0	0.0
6	55.4	4.9	73.4	12.1	113.6	49.2
10	43.6	8.8	49.4	26.2	58.9	41.8
14	33.3	10.1	22.3	24.7	40.4	41.0
18	28.6	11.9	15.3	29.4	36.4	47.4
22	24.6	12.4	6.8	26.3	13.3	38.6
30	13.2	9.2	2.9	25.5	2.3	26.9
38	11.2	10.6	0.0	19.7	0.0	13.3
60	2.2	8.4	0.0	5.8	0.0	4.5

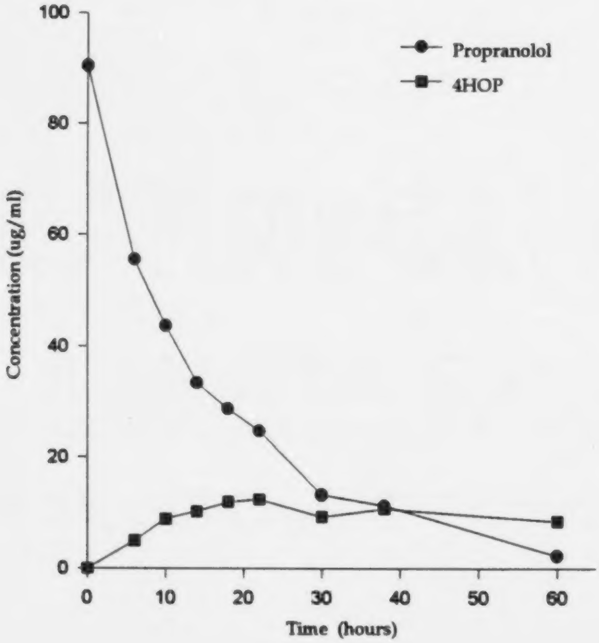


Figure 5-7. A concentration time profile of propranolol and 4HOP in **yeast extract broth** (pH 7.0) following the aseptic addition of 90.4 µg/mL of *rac*-propranolol.

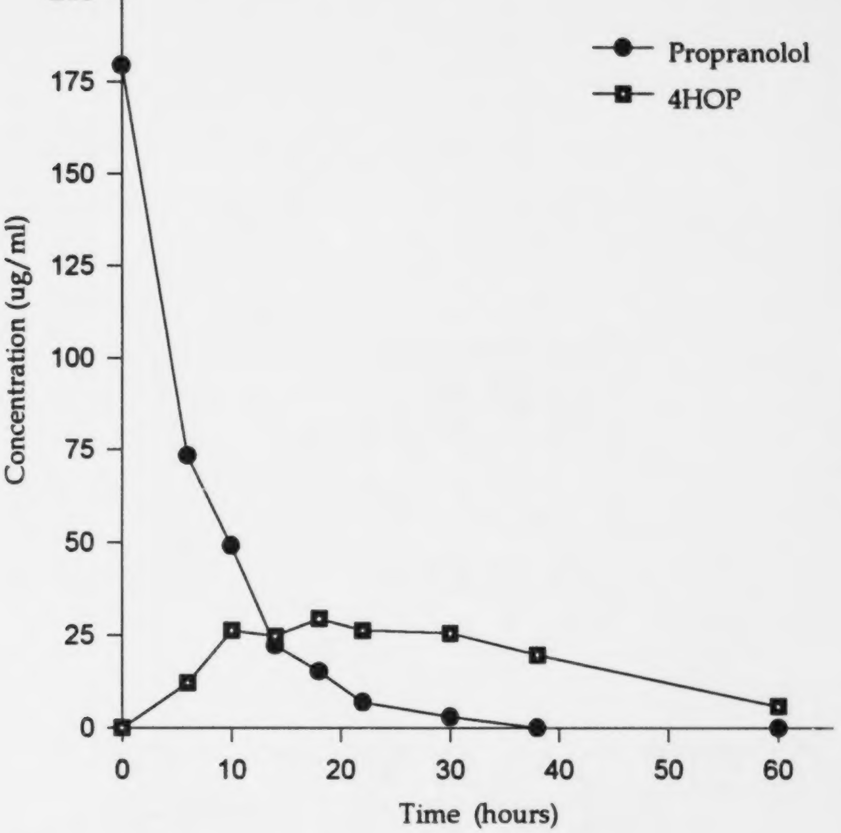


Figure 5- 8. A concentration time profile of propranolol and 4HOP in **yeast extract broth** (pH 7.0) following the aseptic addition of 179.4 μg/mL of *rac*-propranolol.

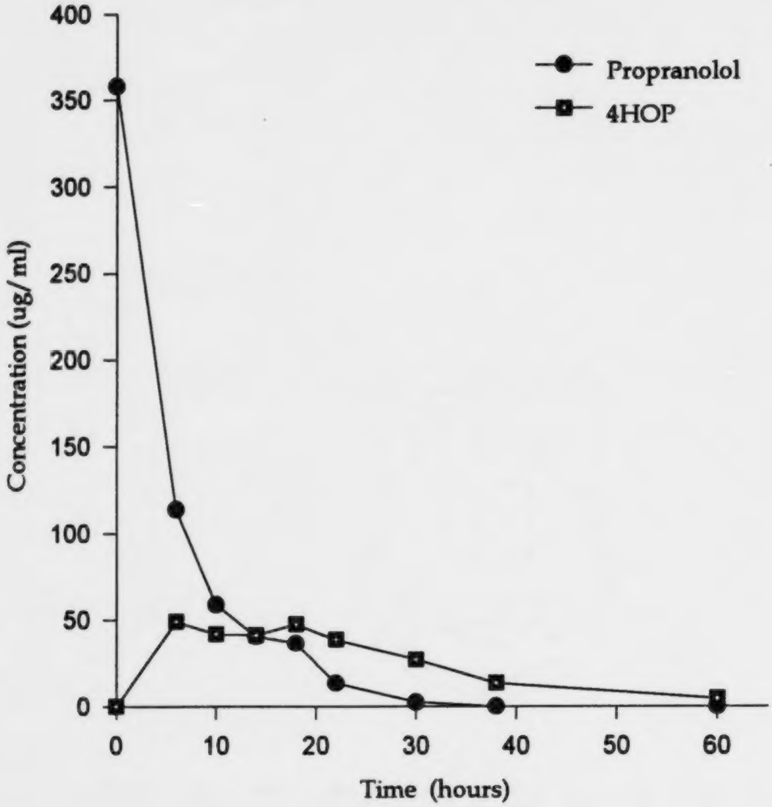


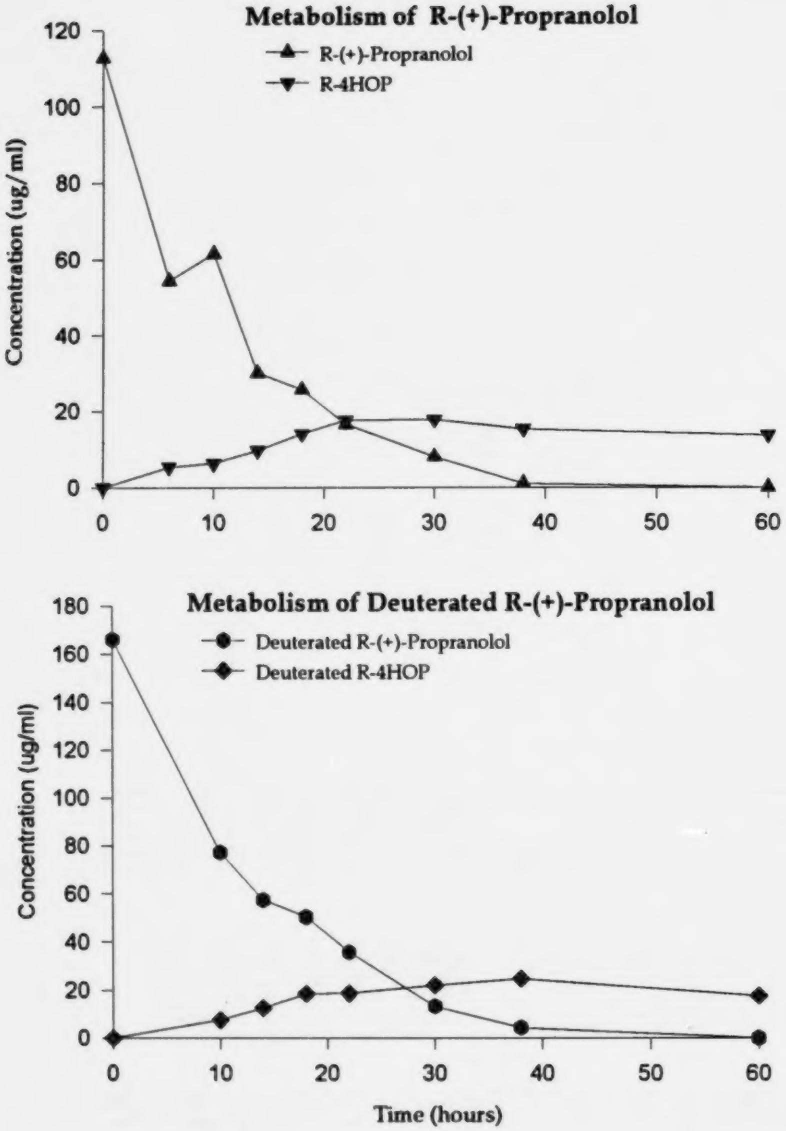
Figure 5-9. A concentration time profile of propranolol and 4HOP in **yeast extract broth** (pH 7.0) following the aseptic addition of 358.0 µg/mL of *rac*-propranolol.

5.2.3 Biosynthesis of Deuterated Metabolites From a Deuterated Substrate.

An advantage of fungal models of mammalian metabolism is the ability to scale-up biosynthesis of a metabolite for additional pharmacological evaluation or structure elucidation. This observation provided the basis for studying 4HOP because our goal was to optimize and increase its production using *C. echinulata* 4145. Accordingly, by utilizing optimal conditions identified with *rac*-mexiletine metabolism, we attempted to maximize 4HOP output. In addition, the metabolism of *rac*, R-(+), and S-(-)-propranolol was compared with that of deuterated *rac*, R-(+), and S-(-)-propranolol. As the deuterium label (position 4) was in a metabolically labile part of the molecule, it was important to determine whether conversion to deuterated 4HOP was hindered (Tables 5-3 to 5-6 and Figures 5-10 to 5-13).

Table 5-3. Comparison of the concentrations of R-(+)-propranolol and R-4HOP with deuterated R-(+)-propranolol and deuterated R-4HOP in **yeast extract broth** at pH 7.0.

R-(+)-Propranolol			Deuterated R-(+)-Propranolol		
Time (hours)	R-(+)-Propranolol (μg/mL)	R-4HOP (μg/mL)	Time (hours)	R-(+)-Propranolol (μg/mL)	R-4HOP (μg/mL)
0	112.6	0.0	0	166.2	0.0
6	54.3	5.3	10	77.3	7.4
10	61.4	6.2	14	57.3	12.4
14	30.0	9.6	18	50.3	18.5
18	25.6	14.5	22	35.7	18.6
22	16.5	17.5	30	13.4	22.0
30	7.9	17.7	38	4.3	24.6
38	1.0	15.2	60	0.0	17.5
60	0.0	13.6			



Metabolism of R-(+)-Propranolol

Metabolism of Deuterated R-(+)-Propranolol

Figure 5-10. Concentration time profiles of R-(-)-propranolol, R-4HOP, deuterated R-(-)-propranolol, and R- deuterated 4HOP in yeast extract broth (pH 7.0) following aseptic addition of 112.8 and 166.2 μg/mL of *rac*-propranolol and deuterated *rac*-propranolol, respectively.

Table 5-4. Comparison of the concentrations of S-(-)-propranolol and S-4HOP with deuterated S-(-)-propranolol and deuterated S-4HOP in **yeast extract broth** at pH 7.0.

S-(-)-Propranolol			Deuterated S-(-)-Propranolol		
Time (hours)	S-(-)-Propranolol	S-4HOP	Time (hours)	S-(-)-Propranolol	S-4HOP
0	109.5	0.0	0	120.4	0.0
6	54.3	7.8	6	86.9	7.6
10	28.7	14.3	10	64.4	15.3
14	18.0	18.7	14	44.9	19.2
18	9.8	19.5	18	32.3	23.5
22	4.8	17.6	22	15.6	21.9
30	1.3	15.8	30	6.1	27.7
38	0.0	10.2	38	1.3	26.0
60	0.0	3.8	60	0.1	12.9

Table 5-5. Comparison of the concentrations of propranolol and 4HOP with deuterated propranolol and deuterated 4HOP in **yeast extract broth** at pH 7.0.

rac-Propranolol			Deuterated *rac*-Propranolol		
Time (hours)	Propranolol	4HOP	Time (hours)	Propranolol	4HOP
0	269.8	0.0	0	200.1	0.0
6	145.2	26.6	6	173.9	10.1
10	73.1	46.5	10	123.0	22.6
14	35.4	51.5	14	83.8	35.1
18	15.8	52.1	18	43.4	42.4
22	6.0	42.8	22	16.4	43.1
30	0.7	28.5	30	2.4	33.9
38	0.0	21.3	38	0.0	28.7
60	0.0	8.5	60	0.0	17.6

Table 5-6. Comparison of the concentrations of propranolol and 4HOP with deuterated propranolol and deuterated 4HOP in **yeast extract broth**, at pH 7.0, in 250 mL Erlenmeyer flasks.

rac-Propranolol			Deuterated *rac*-Propranolol		
Time (hours)	Propranolol	4HOP	Time (hours)	Propranolol	4HOP
0	269.8	0.0	0	200.1	0.0
6	75.8	13.4	6	137.4	22.0
10	46.3	27.1	10	68.8	32.1
14	25.6	37.9	14	47.1	42.0
18	11.7	38.1	18	31.3	50.0
22	3.0	26.6	22	21.0	54.4
30	0.0	22.5	30	6.3	55.3
38	0.0	15.3	38	1.3	40.3
60	0.0	4.1	60	0.5	26.5

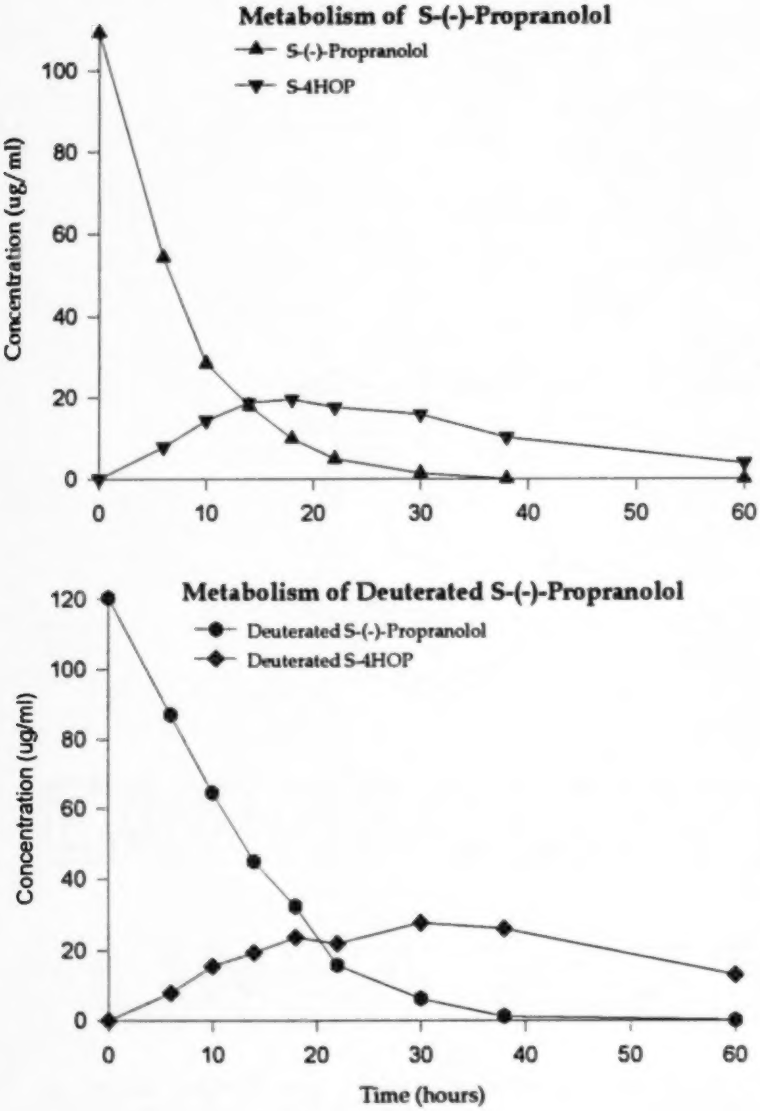
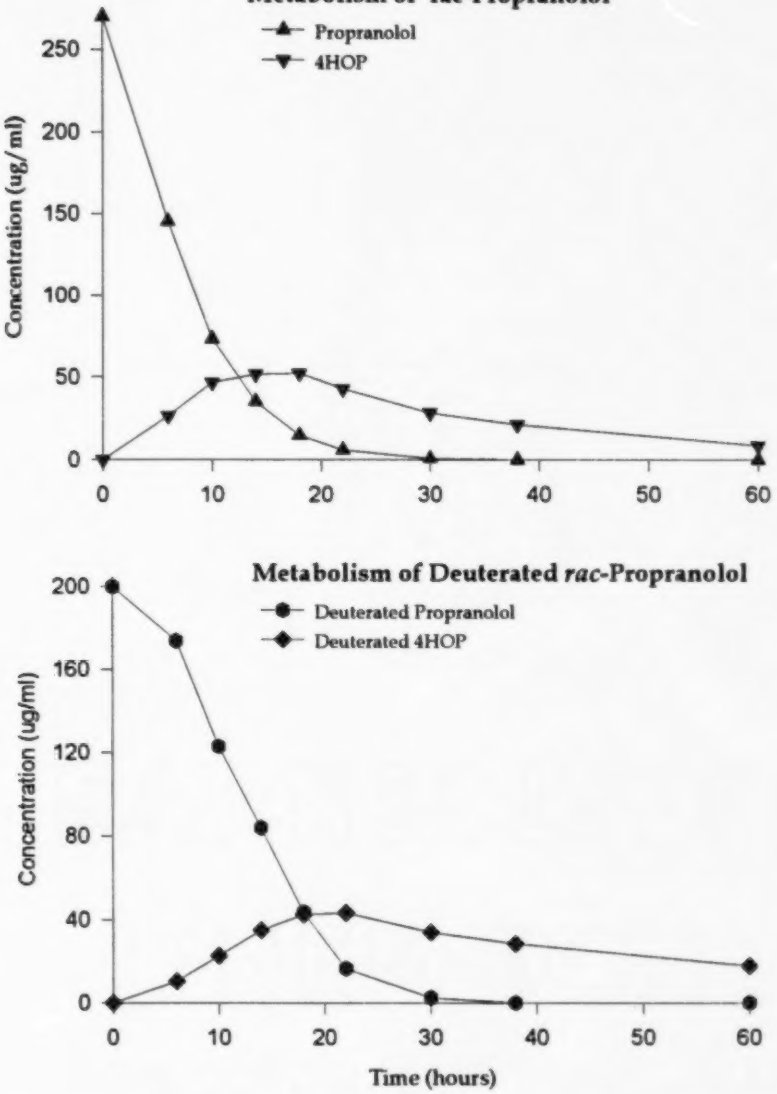


Figure 5-11. Concentration time profiles of S-(-)-propranolol, S-4HOP, deuterated S-(-)-propranolol, and S- deuterated 4HOP in **yeast extract broth** (pH 7.0) following aseptic addition of 109.5 and 120.4 μg/mL of *rac*-propranolol and deuterated *rac*-propranolol, respectively.



Metabolism of rac-Propranolol

Metabolism of Deuterated *rac*-Propranolol

Figure 5-12. Concentration time profiles of propranolol, 4HOP, deuterated propranolol, and deuterated 4HOP in yeast extract broth (pH 7.0) following aseptic addition of 269.8 and 200.1 μg/mL of *rac*-propranolol and deuterated *rac*-propranolol, respectively.

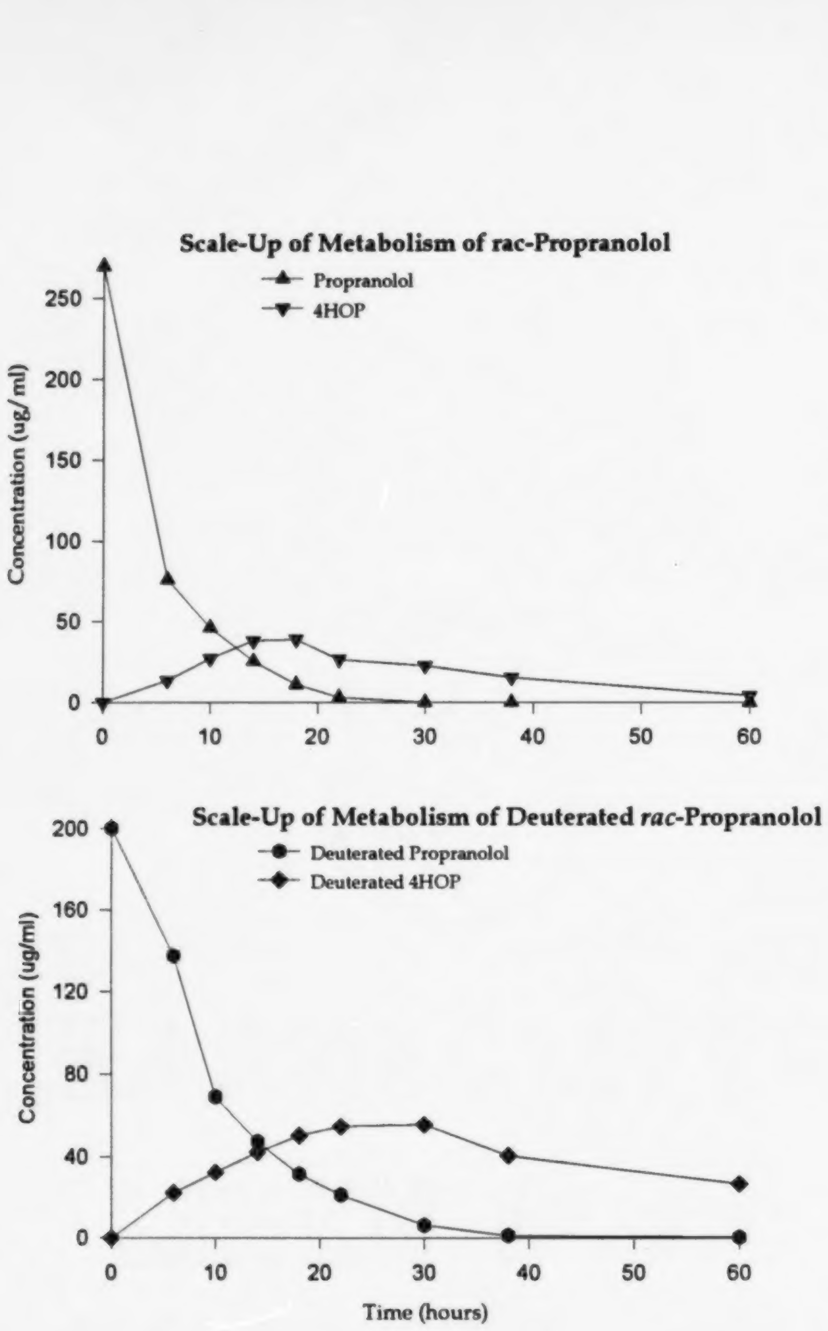


Figure 5-13. Concentration time profiles of propranolol, 4HOP, deuterated propranolol, and deuterated 4HOP in yeast extract broth (pH 7.0) under scale-up conditions (250 mL Erlenmeyer flask) following aseptic addition of 269.8 and 200.1 µg/mL of *rac*-propranolol and deuterated *rac*-propranolol, respectively.

5.2.4 Utilizing Czapec Dox Broth for Phase II Growth

It was determined in the previous chapter that fungal growth was an important factor in biotransformation reactions, and the suggestion was made that if isolating metabolites from a complex nitrogen source was difficult, then a minimal medium could be used to help simplify the process. The following experiment was designed to test this theory by utilizing yeast extract broth for phase I and czapec dox broth for phase II of a biotransformation experiment. The substrates tested were *rac*-propranolol, deuterated *rac*-propranolol and deuterated S-(-)-propranolol (Table 5-7 and Figure 5-14).

Table 5-7. A concentration profile of *rac*-propranolol and *rac*-4HOP in **czapec dox broth** at pH 7.0 (phase II) following initial growth in yeast extract broth. The substrates utilized are indicated below.

	rac-Propranolol		Deuterated *rac*-Propranolol		Deuterated S-(-)-Propranolol	
Time (hours)	Propranolol (ug/mL)	4HOP (ug/mL)	Propranolol (ug/mL)	4HOP (ug/mL)	S-(-)-Propranolol (ug/mL)	S-4HOP (ug/mL)
0	269.8	0.0	200.1	0.0	120.3	0.0
6	233.9	2.5	86.2	22.0	36.1	6.4
10	153.3	16.9	9.7	33.8	4.4	6.5
14	33.3	36.2	1.42	32.3	0.9	4.8
18	1.9	37.5	0.3	22.5	0.5	4.4
22	0.0	28.8	0.0	23.4	0.0	4.3
30	0.0	19.7	0.0	19.0	0.0	3.9
38	0.0	19.4	0.0	15.0	0.0	3.0
60	0.0	4.4	0.0	9.3	0.0	3.0

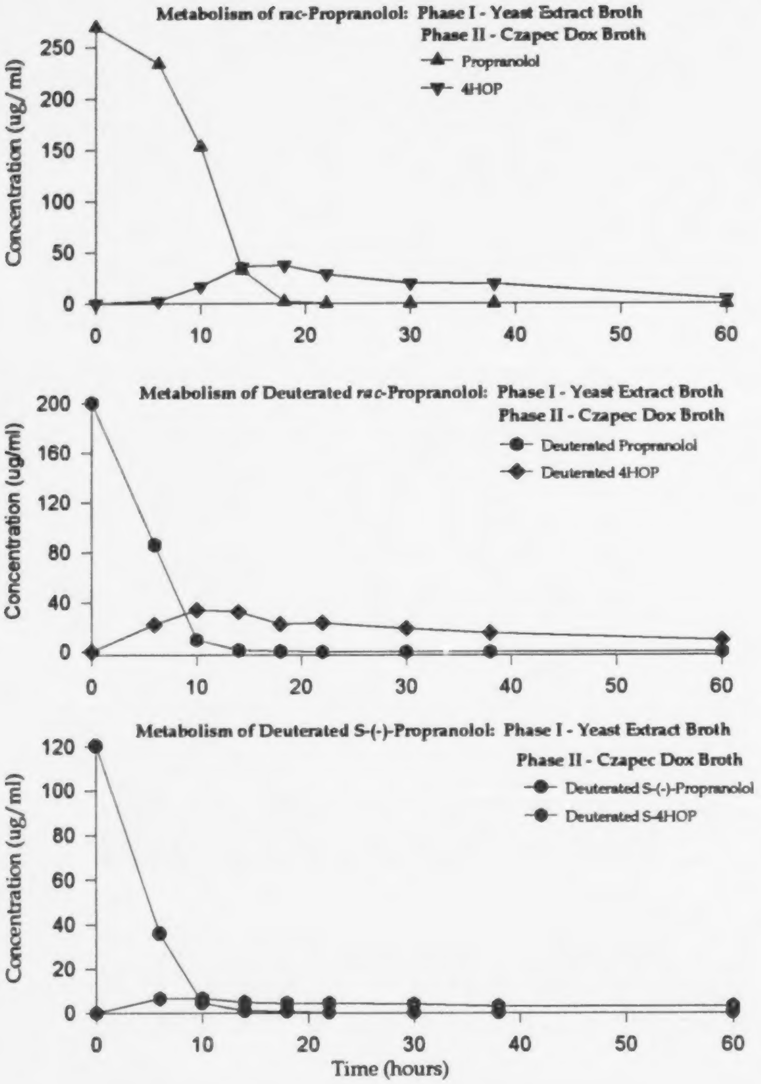
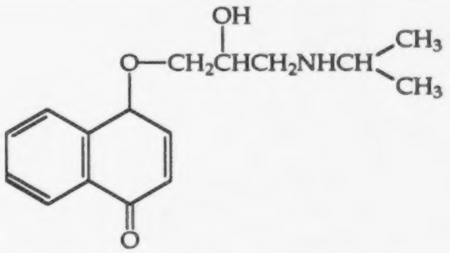


Figure 5-14. Concentration time profiles of propranolol, 4HOP, deuterated propranolol, deuterated 4HOP, deuterated S-(-)-propranolol and deuterated S-4HOP in czapec dox broth, at pH 7.0 (phase II), following initial growth in yeast extract broth.

5.2.5 Effect of Ascorbic Acid on *rac*-4HOP Concentration

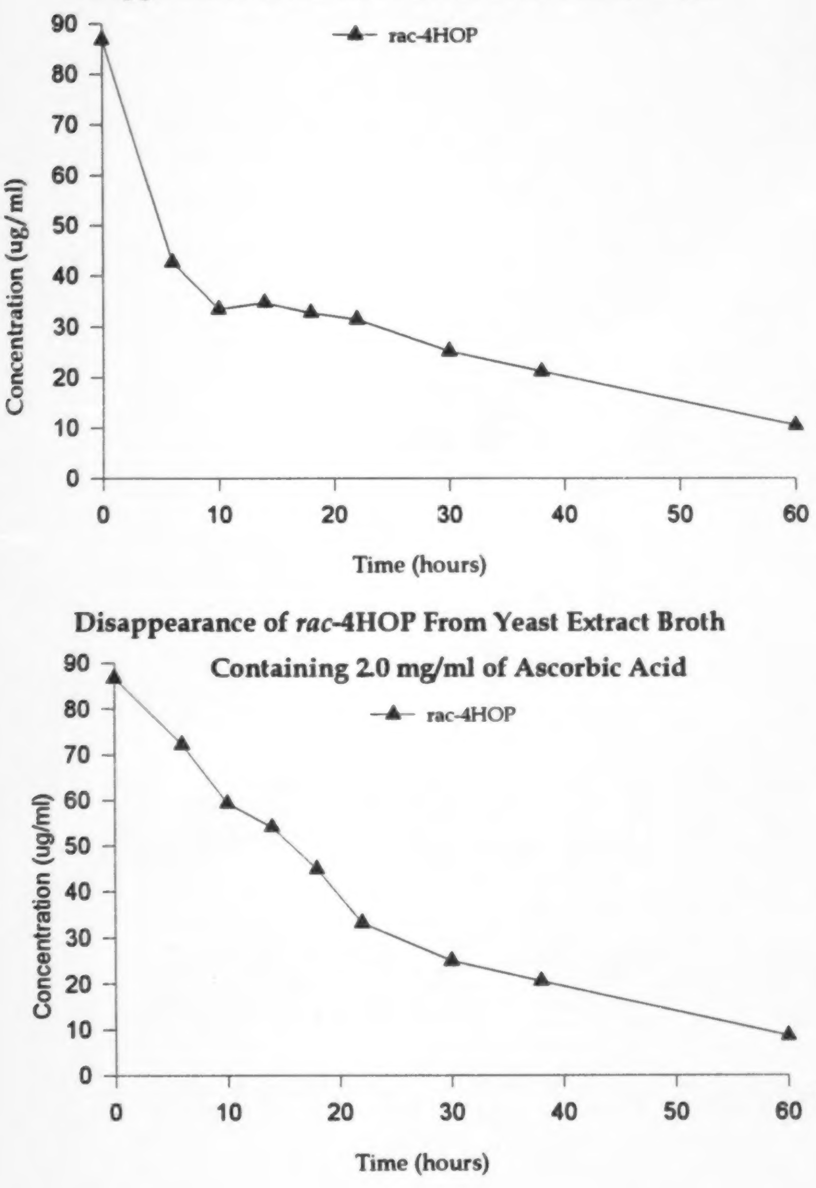
A problem with 4HOP quantitation was its low stability in solution (Walle *et al.* 1975). This can be detected visibly by monitoring a stock solution of 4HOP, over a period of two days, during which time it will turn dark brown. In addition, fermentation media containing *rac*-propranolol and *C. echinulata* 4145 will also turn a dark brown over time suggesting the formation and subsequent degradation of 4HOP. A possible explanation for this color change was the oxidation of 4HOP into a naphthyl enone. Foster *et al.* (1989b) addressed this problem by adding the anti-oxidant ascorbic acid to the growth medium (2.0 mg/mL). The following experiment was constructed to determine the effectiveness of adding 2.0 mg/mL ascorbic to the phase II growth medium (without the fungus).



Naphthyl enone of 4-HOP. A possible structure for the brown pigment.

Table 5-8. Concentration profile of *rac*-4HOP added to yeast extract broth and allowd to incubate under identical conditions used to conduct biotransformation experiments.

	No Ascorbic Acid Added	2.0 mg/mL of Ascorbic Acid Added
Time (hours)	*rac*-4HOP (ug/mL)	*rac*-4HOP (ug/mL)
0	86.8	86.8
6	42.7	72.1
10	33.3	59.4
14	34.6	54.1
18	32.6	44.8
22	31.3	33.2
30	25.1	25.0
38	21.1	20.6
60	10.5	8.5


Disappearance of *rac*-4HOP From Yeast Extract Broth

Disappearance of *rac*-4HOP From Yeast Extract Broth Containing 2.0 mg/ml of Ascorbic Acid

Figure 5-15. A concentration time profile of yeast extract broth containing *rac*-4HOP with and without 2.0 mg/mL ascorbic acid

5.3 Discussion

Studies in this chapter confirmed that C. *echinulata* was capable of metabolizing *rac*-propranolol to the hydroxy metabolite 4HOP. As was seen with *rac*-mexiletine, the nitrogen source utilized had an effect on drug metabolism, which did not appear to be stereoselective. On average, 17.7% of the *rac*-propranolol added to the fermentation medium was converted to 4HOP. In addition, the deuterium label at position 4 of the naphthyl ring did not adversely affect metabolite biosynthesis. However, degradation of the product, 4HOP, was a problem under all growth conditions tested. Furthermore, it appeared that ascorbic acid addition was only capable of decreasing the rate at which degradation occurred rather than prevent it.

Originally, *rac*-propranolol metabolism was to be studied using a stereospecific HPLC method capable of quantifying the diastereomers of propranolol and 4HOP (Chapter 2.5.3). However, the appearance of an unknown metabolite, which was not present in fungal controls, with a retention time similar to that of 4HOP made it impossible to quantify 4HOP diastereomers. Therefore, a non-stereospecific reverse phase HPLC assay was employed for the majority of this study (Chapter 2.5.4). However, studies conducted using the stereospecific assay did provide information regarding *rac*-propranolol metabolism.

It was demonstrated qualitatively that C. *echinulata* 4145 was capable of biosynthesizing 4HOP and this process did not appear to be stereoselective (Figure 5-3). In addition, there was a strong possibility that the interfering metabolite was an hydroxylated compound. There were several factors supporting this hypothesis credibility; these included the similarity in retention time with 4HOP. In addition, in order for the unknown metabolite to appear in the chromatogram, it would have to be extracted at a basic pH (like the phenol 4HOP), have an amine functional group to react with NEIC, and a chiral center.

Therefore, if it was a metabolite of *rac*-propranolol, the propanolamine side chain would have to be intact. This provides circumstantial evidence to support the identy of a hydroxy metabolite.

Data obtained with the stereospecific assay confirmed a previous finding that the nitrogen source employed affected drug metabolism. Propranolol was no longer detected in yeast extract broth after 53 hours, in peptone broth after 30 hours, and trypticase soy broth after 22 hours. The slow uptake of *rac*-propranolol observed with yeast extract broth and the rapid uptake in trypticase soy broth was also found with *rac*-mexiletine metabolism. A noticeable difference, between these two sets of experiments, was the length of time required to remove *rac*-propranolol from peptone broth.

The effect of *rac*-propranolol concentration on metabolism was evaluated, using C. *echinulata* 4145 in yeast extract broth at pH 7.0. It was determined that the amount of drug added influenced metabolite biosynthesis (Table 5-9). As

Table 5-9. A summary of data obtained by adding different concentrations of *rac*-propranolol to yeast extract broth containing C. *echinulata* 4145.

	Concentration of *rac*-Propranolol Added to Each Flask (µg/mL)			
	100	200	300	400
Maximum amount of 4HOP detected (µg/mL).	12.4	29.4	52.1	49.0
Time required to reach maximum concentration (hours)	22	18	18	6
% of drug added.	13.7	16.4	19.3	13.7
Time required to metabolize *rac*-propranolol (hours).	>60	38	38	38

the substrate concentration increased, there was a corresponding increase in 4HOP production (% of drug added) up to a drug concentration of 300 μg/mL. After this point a decrease was observed. In addition, increasing the amount of *rac*-propranolol added decreased the time needed to obtain the maximum concentration. Therefore, adding 300 μg/mL of propranolol was optimal for *rac*-4HOP biosynthesis.

Based on this initial data an experiment was designed to demonstrate how fungal models could be used to generate additional information regarding the metabolism of new therapeutic agents. This involved the use of isotopically labeled *rac*-propranolol to yield similarly labeled metabolite. If sufficient quantities of metabolite could be produced, this technique could be exploited to generate labeled metabolites for additional metabolic evaluation. The deuterium labeled racemic, R-(+), and S-(-)-propranolol was synthesized by Kevin Morin, a fellow graduate student (Chapter 2.7). A summary of results obtained with these compounds, and the corresponding unlabeled substrates is presented in table 5-10.

Table 5-10. A summary of data obtained by utilizing deuterium labeled and unlabeled *rac*-propranolol as substrate.

	Substrate Added					
	R-(+)-Propranolol		S-(-)-Propranolol		*rac*-Propranolol	
	Unlabeled	**Labeled**	**Unlabeled**	**Labeled**	**Unlabeled**	**Labeled**
Maximum amount of 4HOP detected (μg/mL).	17.8	24.6	19.6	27.8	52.1	43.1
Time required to reach maximum concentration (hours).	30	38	18	30	18	22
% of drug added.	15.8	14.8	17.8	23.1	19.3	21.6
Time required to metabolize *rac*-propranolol (hours).	60	60	38	60	38	38

The data indicated that addition of a deuterium label to positions 2 and 4 of the naphthyl ring did not prevent 4HOP biosynthesis. Furthermore, a higher percent conversion of labeled 4HOP and S-4HOP was observed when compared to unlabeled 4HOP and S-4HOP production. The percent conversion, which is the concentration of metabolite produced divided by the amount of drug added multiplied by 100, was used for all comparisons. This number was required because a different concentration of drug was added to each flask making direct comparisons difficult. In addition, there was a higher conversion of S-(-)-propranolol to S-4HOP, as a percent of drug added, than was observed with R-(+)-propranolol. The increase was 12 % for unlabeled S-4HOP and 32% for labeled S-4HOP.

In general, when a comparison was made between the times needed to reach maximum metabolite concentration, it was observed it took longer to produce labeled 4HOP than unlabeled compound. This increase could be attributed to the deuterium atoms. One deuterium was attached to a metabolically labile site raising the possibility it could be interfering with cytochrome P_{450} oxidation. However, the kinetics of this reaction would have to be examined, using purified enzyme, to substantiate this. The possibility also exists that deuterium could be hindering transport of the drug, and metabolite, in and out of the cell.

A difference was also detected in the conversion percentages of R and S-4HOP. More substrate was converted into S-4HOP than R-4HOP. The R/S ratio, of percent conversion, for labeled drug was 0.89 and 0.64 for unlabeled 4HOP. These ratios indicate that fungal metabolism does not follow the human preference for R-(+)-propranolol.

An attempt was made to scale up the production of 4HOP and deuterated 4HOP using 250 mL Erlenmeyer flasks. The concentrations of *rac*-propranolol, and deuterium labeled *rac*-propranolol, added to these flasks was the observed optimal concentration of 300 μg/mL. The maximum concentration of 4HOP produced was 37.98 μg/mL (18 hours) which was 14.4 % of the added drug. The maximum amount of labeled 4HOP biosynthesized was 55.33 μg/mL (30 hours) which was 27% of added drug. This converts to 2.77 mg of deuterium labeled 4HOP in one 250 Erlenmeyer flask. To put this value into perspective, 20 such flasks were routinely used for biotransformation experiments. Therefore, taking into account phase I and II growth, it would be possible to generate 50 mg of deuterium labeled *rac*-4HOP in 4 days. In addition, the potential existed to scale up this reaction even further.

A problem not addressed with scale-up was product isolation and purification. Even though 50 mg of labeled *rac*-4HOP could theoretically be produced, isolating it from a complex fermentation medium would be difficult. An approach that addressed this problem was the utilization of a simplified medium for phase II growth. *C. echinulata*'s ability to metabolize *rac*-propranolol, as well as labeled racemic and labeled S-(-)-propranolol was evaluated using czapec dox broth after phase I growth was generated in yeast extract broth. A summary of these results can be seen in table 5-11.

The data indicated that this procedure could be utilized to generate *rac*-4HOP. However, the concentration of metabolite produced was less than that observed with the use of yeast extract broth for phase II. More specifically, when directly compared to production in yeast extract broth, 72% 4HOP, 78% deuterated 4HOP, and 23% S-deuterated 4HOP were biosynthesized in czapec dox broth. When the amount of metabolite produced in czapec dox broth was compared to results obtained with *rac*-mexiletine metabolism in phosphate buffer, PHM biosynthesis was 62% of that obtained with trypticase soy broth. Consequently, this approach had the potential for becoming a means for simplifying product isolation. As was mentioned in chapter 4, czapec dox broth can provide a backdrop to test specific chemicals (e.g. vitamins) for their ability to improve yield. Therefore, using this approach, the potential still exists to improve labeled 4HOP biosynthesis.

Table 5-11. Summary of data obtained when czapec dox broth was utilized for phase II biotransformation.

	Substrate Added to Each Flask		
	rac-Propranolol	Labeled *rac*-propranolol	Labeled S-(-)-propranolol
Maximum amount of 4HOP detected (µg/mL).	37.5	33.9	6.6
Time required to reach maximum concentration (hours).	18	10	10
% of drug added.	13.9	16.9	5.5
Time required to metabolize *rac*-propranolol (hours).	22	22	18

Finally, a problem encountered under all fermentation conditions was that the concentration of 4HOP in the growth medium slowly decreased over time. This phenomenon could be reproduced in control reactions that did not contain fungus. It has been reported that *rac*-4HOP underwent further oxidation (Foster *et al.* 1989b) which turned the fermentation medium and mycelia into a dark brown color. It was also reported that adding ascorbic acid to the fermentation medium would prevent this. However, figure 5-14 clearly shows that ascorbic acid addition will only decrease the rate of 4HOP disappearance. Therefore, a future experiment could try other antioxidants. In addition, it would be possible to take advantage of C. *echinulata*'s ability to metabolize drugs at a low concentration of dissolved oxygen. This might help to improve the stability of *rac*-4HOP in solution.

5.4 Conclusion

Using the substrates R-(+), S-(-), *rac*-propranolol, and isotopically labeled *rac*-propranolol, it was demonstrated that *C. echinulata* 4145 was capable of biosynthesizing the major human metabolite 4-hydroxypropranolol. The optimal *rac*-propranolol concentration for 4HOP biosynthesis in yeast extract broth was 300 μg/mL. By utilizing a deuterium labeled substrate, biosynthesis of a deuterium labeled 4HOP metabolite was achieved. Finally, the minimal medium czapec dox broth could be utilized for metabolite biosynthesis. This simplified growth medium was employed to aid with product isolation.

6.1 Final Summary

This work illustrates how fungal models of mammalian metabolism can provide useful information regarding the oxidative biotransformation of xenobiotics. More specifically:

1. They provide a simple and inexpensive means of generating an oxidative metabolic profile.

2. By utilizing standard protocols (listed in methods and materials) reproducible fungal growth can be generated capable of biotransforming xenobiotics of interest. In addition, an autoclave and incubator are the only specialized equipment required.

3. Fungal biosynthesis can be scaled up to yield (semi)-preparative amounts of metabolite for additional pharmacological evaluation and structure elucidation.

4. By using isotopically labeled drug substrates (semi) preparative amounts of labeled metabolite can be produced.

5. Medium composition and pH have a large effect on the quantity and composition of metabolites biosynthesized in a biotransformation experiment.

6. Metabolism occuring in the fungal model is stereoselective.

7. Fungi are capable of biosynthesizing phase II metabolites.

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